ANALYSIS OF BACTERIAL POPULATION AND DISTRIBUTION IN THE DEVELOPING STRATA OF A CONSTRUCTED WETLAND USED FOR CHLORINATED ETHENE BIOREMEDIATION

THESIS

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Abstract

Chlorinated hydrocarbons and their degradation products are among of the most common organic groundwater contaminates in the United States. These compounds attack the central nervous system in animals and can affect the photosynthesis of plants. These compounds are also resistant to degradation in the environment and, because of this, pose a risk to any ecosystem in which they are present.

This study identified the dominant microbial species in a constructed treatment wetland at Wright-Patterson AFB, Dayton, Ohio using 16S rRNA gene sequence analysis. Samples were taken from three different depths and during each of the four seasons. These samples were compared with similar samples taken from an uncontaminated, control site located at Valle Greene wetland in Beavercreek, Ohio. The intent of the study was to measure differences between the microbial community of the treatment wetland and the control wetland. It was hypothesized that the bacteria found to degrade the materials in the lab would be present in the treatment wetland and has a higher population than a wetland free of contaminants. This hypothesis would help support the idea that the natural attenuation of chlorinated hydrocarbons is due primarily to biological factors. The study found that the diversity of microbial communities in both the treatment wetlands and control were so great that additional sampling and sequencing was needed in order to gain a sampling size large enough to establish statistical significance. Over four hundred individual samples were taken and over seventy percent of the organisms from those samples were unique. This was found in both the treatment wetland and the Valle Greene control site.

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ANALYSIS OF BACTERIAL POPULATION AND DISTRIBUTION IN THE DEVELOPING STRATA OF A CONSTRUCTED WETLAND USED FOR CHLORINATED ETHENE BIOREMEDIATION

I. Introduction

Overview

The purpose of this study was to identify the seasonal vertical distribution of dominant bacterial species in the soil of the Wright Patterson Air Force Base constructed wetland and to compare the populations to samples from a natural, uncontaminated wetland located at Valle Greene in Beaver Creek, OH. The dominant species were identified using the 16s rRNA PCR and sequence analysis. The calendar year was divided into four seasonal periods. One core sample was taken from each wetland during each season.

Background

Historically, because of relaxed or negligent disposal practices of chlorinated compounds many of these solvents have seeped into local sub-surfaces thereby contaminating local groundwater supplies. Many of these chlorinated compounds are classified as carcinogens and have been shown to have varying negative effects on humans. It has long been known that tetrachloroethylene (PCE) and trichloroethylene (TCE) are toxic and that a degradation product, vinyl chloride (VC), is a carcinogen (Dougherty, 2000). Recent investigations focusing on the kinetics, metabolism, and

toxicology of two metabolites of TCE, dichloroacetate (DCA), and chloral hydrate have proven these compounds to be potential endocrine disruptors (Cornett et al., 1999). The main effects of PCE in humans are neurological, liver, and kidney effects following acute (short-term) and chronic (long-term) inhalation exposure (U.S. EPA, 1988). Epidemiological studies of dry-cleaners occupationally exposed to tetrachloroethylene suggest increased risks for several types of cancer. Animal studies have reported an increased incidence of liver cancer in mice, via inhalation and gavage (experimentally placing the chemical in the stomach), and kidney cancer and mononuclear cell leukemia in rats (U.S. EPA, 1988).

Chlorinated solvents have also been identified to be among the most common groundwater contaminants in the United States. Concerns that contaminated groundwater could emerge as surface water is also a threat due to site hydrogeology and topography. Because of these combined issues, many of these contaminated sites fall under the requirements of the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). CERCLA, also known as Superfund, created by Congress in the 1980s, was intended to address concerns about the clean up of uncontrolled and/or abandoned waste sites that could be hazardous to humans, the environment, or could become possible future hazards. The National Contingency Plan, which is an identified remediation process within CERCLA, requires the development of a plan for allocating resources in order for companies and government agencies to properly manage their environmental compliance. This compliance can be both costly and time consuming. Following is a list from the National Priorities List (NPL) that ranks contaminated sites and the types of contaminates. Table 1 summarizes the overall list found in the study.

Table 1. Chlorinated VOCs Frequencies of Occurrence

NPL Ranking	Name	NPL Site Frequency
4	Vinyl chloride (VC)	608
16	Trichloroethylene (TCE)	1021
30	Tetrachloroethylene (PCE)	930
43	Carbon Tetrachloride	422
87	1,2-Dichloroethane	599
148	1,1,2,2-Tetrachloroethane	327
163	1,1,2-Trichloroethane	274
175	1,2-Dichloroethene, Trans-	598
213	1,2-Dichloroethylene	450
277	1,2-Dichloroethene, Cis-	263
282	Dichloroethylene (DCE)	114

Source: (U.S. Department of Health and Human Services. Agency for Toxic Substances and Disease Registry, 2003). Ranking based on combination of toxicity, frequency, and potential for human exposure.

The EPA (2004) reports that 69 percent of the NPL sites are contaminated with halogenated VOCs. Similarly, halogenated VOCs are by far the most common contaminant at Resource Conservation and Recovery Act (RCRA) sites, found at 60 percent of the sites (U.S. EPA, 2004). Unfortunately, chlorinated compounds are persistent in soils and therefore degrade slowly. This slow degradation requires significant man-hours that translate into potentially economic burdens and significant health hazards.

The federal government and private industry are investigating the use of treatment wetlands to degrade hazardous chemicals into non-hazardous by-products. Companies and agencies are no longer left unaccountable for their practices with the current regulation of chemical production, use, and disposal; they have a social responsibility to use sustainable development, which requires a minimal environmental footprint. This is why the concept of a treatment wetland has both environmental and economic appeal. A

treatment wetland can offer both a habitat and provide a passive method for cleaning up waste products produced and used by companies or governmental agencies. This process can be used to clean up existing wetlands or a treatment wetland can be constructed to treat a continuous source of contaminates. This process is known as bioremediation and its success is based on a combination of parameters, including the impact of subsurface microbial activity, vegetation interactions, organic matter (OM), and physical factors such as groundwater flow. Subsurface microbial activity is the focus of this report and is specifically referred to as natural attenuation. Contaminants in soil and groundwater must be “bioavailable” to be remediated (absorbed, modified, degraded, transformed, sequestered, etc.) by either plants or microorganisms (Shimp et al., 1993). The groundwater and dissolved contaminants move through the rhizosphere (zone of soil that surrounds and is affected by the roots of plants), where they are subjected to bioremediation by microorganisms and soil interactions before entering plant roots. In some instances, the magnitude of microbial transformation of TCE can be significantly larger than plant influence (Anderson and Walton, 1995) although this depends on the site and plants used (Nichols et al., 1997, Schnabel et al., 1997). It is not currently known how the microbial community makeup affects the remediation process of contaminates.

Definition of a Microbial Community

As used here, "microbes" includes bacteria, viruses, yeasts, and microscopic fungi. In wetlands, these have most often been measured indirectly, in the pursuit of estimates of microbe-related processes relevant to element cycling, such as

decomposition and de-nitrification. Although microbial responses to contaminants have been summarized for other surface waters (e.g., Cairns et al., 1972) and upland soils (Baath, 1989), few studies have looked at microbial community structure specifically in wetlands, or identified particular microbes as indicators of wetland ecological conditions. (http://www.epa.gov/owow/wetlands/wqual/microb.html)
EPA Report # EPA/600/3-90/073

Microbial Diversity

Dominant species may or may not be the keystone of a community regarding the flow of energy or nutrients, but because dominant species often achieve their status at the expense of other species in the community, they tend to be the controlling factor in the local ecological system (Smith, 2003). There are numerous methods for identifying the dominant species within a community. The first step in identifying a dominant species is to specify the metric used. This metric can be based on a species dominant factor of: species biomass, species occupied count, species contribution to the energy flow, or species control over the community. Concerning the microbial community, diversity is a difficult statistic to calculate with any chosen factor (Curtis, 2004).

The species diversity is an indicator of a community's health and ability to adapt to changes in its environment. The diversity of a population is made up of three factors; number of species, species richness, and species evenness (Smith, 2003). The species richness is simply the count of different species or types within a community. The species evenness is the distribution by which a community's number of individuals is grouped among that community's species richness. In most phylotype analysis, it is not

practically feasible to identify all the species. This is primarily due to the fact that genetic libraries are extremely difficult to replicate because of the varying degrees of factors that affect a microbial community's diversity that change between sampling (Curtis, 2004). It has become more accepted to use an abundance-based analysis to show community's diversity as opposed to an incidence-based richness of diversity. The best estimator was found to be S_{Chao1} (Kemp, 2004). S_{Chao1} (Chao 1984, 1987) is a non-parametric estimator that is calculated as

$$S_{Chao1} = S_{obs} + \frac{F_1^{\,2}}{2(F_2 + 1)} - \frac{F_1 F_2}{2(F_2 + 1)^2} \qquad (1)$$

where S_{obs} is the number of phylotypes observed and F_1 and F_2 are the number of phylotypes occurring either one or two times. It is particularly appropriate for data sets in which most phylotypes are relatively rare (Chao, 1987). An alternative estimator for coverage of non-parametric proportions of phylotypes in smaller libraries that represent libraries of infinite size is Good's estimator using the following formula:

$$C = 1 - \frac{n_1}{N} \qquad (2)$$

where n_1 is the number of phylotypes appearing only once and N is the library size (Kemp, 2004). Another coverage estimator by Chao uses the C_{ACE} to represent the non-parametric, abundance-based coverage estimator for relatively low phylotypes that occur less then ten (10) times.

$$C_{ACE} = 1 - \frac{F_1}{N_{rare}} \qquad (3)$$

Where F_1 is the number of phylotypes that only occur once in the sample library and those that occur fewer than ten (10) times are represented by N_{rare} (Kemp, 2004).

16s PCR DNA Analysis

If researchers are not able to recognize and identify individual organisms, then the researchers are unable to establish relationships and links. Historically, biologists have used methods to categorize and identify different species in the environment. This process of categorizing species is necessary in order to allow the repeatability and expansion of biological research. The main accepted method of categorizing different species is referred to as the Linnean system of binomial nomenclature. Microbiologists have historically used this method to categorize microbial organisms. One of the main limitations to this method is that it relies on the observation of organisms that interbreed with each other in order to define a population. Many of the microbial organisms reproduce through cell division and no interbreeding occurs, so grouping organisms into anything smaller than a species becomes virtually impossible.

Seasonal Microbial Communities

Thermal Alteration. Although microbial communities are highly sensitive to temperature, few studies have directly examined the effects of thermal stress on community structure in wetlands.
(http://www.epa.gov/owow/wetlands/wqual/microb.html)
EPA Report # EPA/600/3-90/073

Problem Statement

Contamination of ground and surface water by chlorinated compounds like PCE and TCE are common and are harmful to animals and humans. Chlorinated compounds

do not degrade spontaneously. Treatment wetlands are able to enhance degradation of chlorinated compounds. Both plants and microorganisms can help remediate contaminated soils. Researchers have identified species of microbes that degrade chlorinated compounds in the lab (Bragley, 1990). These same organisms have been shown to be present in the wild where remediation is occurring (Kovacic, 2003). Current research is lacking the link between what is seen in the controlled lab experiments and what is actually occurring in the wild. Interactions of microbes with plants and other microbes, and their effect on the remediation process are not known. The purpose of this thesis is to identify the dominant species present in a treatment remediation wetland using DNA analysis and compare them to an uncontaminated control wetland.

Research Objectives

The objectives of this research were to answer the following questions:

1) What are the dominant microbial species in a treatment wetland?

2) Are there differences in the dominant species in relation to depth?

3) Are there seasonal differences in the dominant species?

4) Are there differences in the dominant species between a site that is contaminated with chlorinated compounds and one that is not?

II. Literature Review

Overview

Among of the most common ground water contaminants in the United States are chlorinated ethenes (McCarty, 1996). The traditional methods for cleaning and restoring these waters, pump and treat, are time consuming and expensive, costing tens of millions of dollars for a single site (Masters, 1997). There are over 7000 sites identified and classified by the Department of Defense as being contaminated with chlorinated aliphatic compounds and the cost estimate for clean up is in the billions (NRC, 1997). Groundwater remediation technologies are used to treat groundwater that has been contaminated, often due to solvents that contain hydrocarbons. The "pump and treat" method of remediation pumps the water to the surface and treats it by using air stripping. Air stripping works on volatile compounds, but for ionic contaminants, reverse osmosis is required. Both these methods are expensive because of the high energy and maintenance required. These methods are not sustainable and new alternatives must be found and proven.

A potential cheaper and more rapid method is the use of microorganisms bioremediation. Identifying the microbial processes by which ground water remediation works is crucial for the improvement of the processes and increased application of the processes. Current understanding of bioremediation allows for only a systemic approximation of the processes, which contribute to the degradation of contaminants. The purpose of breaking these toxic materials into other compounds and elements is to lessen the threat to surrounding ecological systems. This type of remediation is crucial in the development of a sustainable process application, which is needed to meet the present

generation's needs without compromising the abilities of future generations. This process is essential if we are to continue to prosper on this planet and it can only be harnessed if we understand how each part in the system works. One of the major components in the remediation machine is the microbe and the makeup of its community.

Prior studies have shown that co-metabolic reductive dechlorination of chlorinated products have been reduced under sulfate-reducing conditions and that microbial processes are suspected due to the presence of organic acids (Kovacic, 2003). It has not been identified or proven that a diverse and healthy microbial community exists in the different layers of the treatment wetland and no model has been created to represent the microbial processes.

Microbial Populations Diversity and Biodegradation

Biodegradation of chlorinated ethenes can occur through four primary degradation pathways: energy-yielding oxidations, co-metabolic oxidations, energy-yielding reductions, and co-metabolic reductive dehalogenation (Lee, Odom and Buchanan, 1998). To successfully implement a bioremediation system, microbial pathways must be identified. Once identified, these pathways can be monitored and used as indicators or metrics to enhance the progress of a remediation process. Correlation of controlled laboratory experiments and field operations is because of advances in soil chemistry, soil microbiology, soil physics, geology, bioinformatics and plant physiology (Sylvia and others, 2005). Some current uses of biotechnology are the bioconversion of organic waste and the use of genetically altered bacteria in the cleanup of oil spills. The research into the mechanics and properties that promote the attenuation of chlorinated

hydrocarbons has gained the interests of many researchers. The types of bacteria that are present along with the physiological process that propels the microbial ecological machine forward are significantly different from one population to another (Chapelle, 2001). It is this difference that must be understood in order for bioremediation technicians to repeat and predict the outcomes of treatment wetlands.

Research has shown that natural microbial activity, natural attenuation, will degrade PCE in to simpler byproducts, and that under the right conditions, will be transformed into the harmless materials, chloride, carbon dioxide, and water (Lee and others, 1998).

Studies that have compared microbial communities among wetlands (spatial variation) apparently include only Henebry et al. (1981, 1984) and Pratt et al. (1989). The former study, covering 13 Michigan wetlands over a 5-year period, found a range of 93 to 365 protozoan species; Sorenson's similarity index ranged from 0 to 40, with a mean of 21. The latter study, covering 28 Florida ponds, found a range of 112 to 410 species, with a mean of 338 species in non-artificial ponds. Functional group structure of the resident microbial fauna changed slightly from year to year, but wetlands in the same geographic region and experiencing similar climatic patterns had similar proportions of species in each functional group (Pratt et al. 1989).

Microbial densities can vary by 2 to 5 orders of magnitude between sediments, aquatic plants, and the water column (Kusnetsov, 1970). Another study, which examined only one wetland complex (Okefenokee Swamp, Georgia) reported that microbial biomass in sediment ranged from 1 to 28 micrograms/gram (dry weight) (Murray and Hodson, 1984). A third study from Louisiana reported microbial densities of up to 108

micrograms/gram (dry weight) (Felton et al., 1967).

(http://www.epa.gov/owow/wetlands/wqual/microb.html)

EPA Report # EPA/600/3-90/073

Fortunately, certain microorganisms (*Dehalococcoides*) naturally degrade halogenated compounds (Sylvia, 2005). Thus the employment of known dehalogenating microorganisms has surfaced as a valid procedure for remediation within constructed treatment wetlands. In recent years the scientific community has focused on natural attenuation as a viable and economically sound procedure to remediate polluted sites. This has created a need for an analytical procedure to determine the overall microbial diversity and bacterial dominance in wetland ecosystems (Sylvia, 2005; Kemp, 2003). More specifically, the use of genomic techniques for the identification of microbial consortia (i.e. Polymerase Chain Reaction (PCR)) will be the source for analytical interpretation of the treatment wetland at WPAFB.

Even though the capabilities of certain microbial consortia to degrade PCE and TCE is known, the process is not thought to be limited to a unique or combination of microbial species; thus, the employment of procedures to isolate and identify the dominant species in an environmental ecological system. Once the main species are identified and a statistically significant correlation is made to show a difference in dominant species between normal wetlands and treated wetlands, research can be done on specific microbes or groups of microbes. The issue then becomes how can microbial communities be cataloged and studied.

16s rRNA Gene Sequence Analysis

During the early years of molecular biology research, the standard for identifying bacteria was based on the comparison of morphologic and phenotypic characteristics. The phenotypic descriptions were associated with genetically observable traits rather than genomic type genetics. This method adopted taxonomic standards which were used for higher order organisms that could be easily categorized by reproduction patterns and geographic locations. One of the most recent and useful advances in molecular biology has been the development and understanding of polymerase chain reactions (PCR) (Sylvia, 2005).

The PCR process acts to amplify sequences of DNA from very minute samples that can then be used for isolation and analysis of the DNA. The five items needed to perform a PCR are as follows: a template consisting of only a few molecules of the DNA segment, a set of primers, an enzyme to manufacture copies of DNA, a supply of nucleotides, and a means to process the mixture with a temperature cycling routine. Out of the five requirements for the PCR, the most crucial to correctly develop is the type of primers to use (Drlica, 2004).

During the 1980's the genetic basis for identifying bacteria began to receive acceptance in the microbial field. It was demonstrated that certain segments of DNA were common to most bacteria, the sequences for genes that coded for 5S, 16S, and the 23S rRNA. The most commonly identified genetic sequence used today for categorizing bacteria is the 16S rRNA gene (Clarridge III, 2004). The 16S rRNA gene sequence also serves as a molecular chronometer and has a high degree of conservation between bacteria due to the critical component to cell functions (Woese, 1987). The final

advantage to using the 16S rRNA gene is that it can also provide a marker for evolutionary distance and relatedness of organisms even though its absolute rate of change in the gene sequence is not known (Harmsen and Karch, 2004; Kimura, 1980; Pace, 1997; Thorne and others, 1998). The next step in identifying an organism genetically is to choose an appropriate primer set.

A primer is a short sequence of nucleic acids that is used to start the syntheses of a new strand of DNA. The primers can denote the beginning or ending of a synthesized strand and can be designed for specific applications. The number of documented primer sequences has increased over the last two decades and a number of established primers for sequencing rRNA genes have been made available (DasSarma and Fleischmann, 1995; Elwood and others 1985; Kolganova and others, 2002). Primers that contain inosine residues are able to detect bacteria-specific populations and show a cross section of the diversity by using a broader specificity (Watanabe and others, 2001). The 16S rRNA gene sequence PCR primers E8F and E533R were chosen for their highly conserved positions over a wider specificity and their ability to also be complementary to eukaryote rDNA sequences (Baker and others, 2003).

Constructed Treatment Wetland

Wetlands are some of the most productive and diverse ecosystems in the world because they provide and maintain an abundant supply of water, nutrients, and sunlight. The water is the medium in which organisms live, hunt, and transfer resources. The nutrients are the raw materials necessary for life and are consumed in metabolic processes. The sunlight provides the energy for photosynthesis which leads to the

production of biomass. This biomass eventually dies and becomes decomposing organic matter, which feeds the microbial community. One factor that allows such a high macrophyte concentration is the wetland's ability to provide the nutritional capacity of an aquatic environment and the above water access of plants to sunlight. This above water access greatly increases the amount of light energy available to the plants which promotes the growth in biomass. Another factor that helps wetlands produce an abundance of microbial activity is the water-saturated conditions. The exchange rates of gasses between the decomposing matter and the atmosphere are greatly reduced, this reduction in exchange rates promotes an anaerobic condition that is inefficient and caused a slow rate of decomposition and mineralization. The slow rate of decomposition coupled with the rapid rate of biomass production creates a great deal of organic matter that forms multiple layers of sediment that are favorable for microbial communities (Moshiri, 1993).

Wetlands make up a major feature of the landscape and can found in most populated areas of the world. They are unique because of their hydraulic conditions and the role they play between the dry land and the aquatic systems of our world. Wetlands are referred to "the kidneys of the landscape", and act as sinks and sources for the transformation of chemical, biological, and genetic materials (Mitsch, 2000). The reason why constructed wetlands are used can be attributed to issues of control and predictability. The current understanding of the processes of bioremediation is too incomplete to allow for the effective or efficient use of a natural environment. Not only are the desired outcomes unpredictable, but in the worst cases a productive wetland could be severely damaged by the introduction of toxic materials for degradation purposes

(Richardson and Davis, 1987). It is because of the unpredictability that the constructed treatment wetland becomes useful and advantageous. Constructed wetlands allow for a greater degree of control and allow for the establishment of experimental treatment facilities that have a well-defined composition of substrate, plant types, and monitoring capability.

In the summer of 2000, the Air Force Institute of Technology (AFIT) constructed a treatment wetland located on the Wright-Patterson Air Force Base in Dayton, Ohio. The treatment wetland (TW) was to be a joint study on the natural attenuation of PCE in a contaminated aquifer. The TW was designed as a pump fed, upward flow system that forced contaminated water through a hydric strata that consisted of three separately designed layers. Each stratum is approximately eighteen inches thick with the top layer (section one), consisting of hydric soil characterized by the ability to promote an anaerobic condition when saturated. The middle layer (section two), is iron-rich soil that allows for the generation of Fe^{+3} reducing conditions that promote the degradation of vinyl chloride (Bradley and Chapelle, 1997). The bottom layer (section three), constructed of hydric soil, was mixed with woodchips. The woodchip to soil proportion was 1:8 and was expected to provide an initial source of organic carbon to facilitate microbial growth (Kovacic, 2003).

Organic acids and acetate are essential in the microbial process that allow for the growth and sustainment of diverse microbial populations (Seagren and Becker, 1999). The measurement of these compounds can act as preliminary indicators of biological activity. Prior studies have found that, based on the levels of organic acids, the microbial

communities were dominantly in the top and bottom layers (Seagren and Becker, 1999). The regions support the reductive dechlorination of PCE, TCE, and DCE (Clemmer and Opperman, 2003).

Seasonal Changes

The seasonal periods were identified as spring, summer, winter, and fall. Each core sample was then broken down into three sub samples in order to explore the microbial communities at each level. The different levels are significant because of the difference in measurements of dissolved oxygen, oxidation-reduction potential, temperature and pH levels which may affect the microbial ecology. It was also viewed as an initial step into the establishment of a relationship between the microbial community and growth and the root colonization of the surrounding soil. It was found that organic acid levels taken from identical places at different times of the year, showed substantial flux (Kovacic, 2003). The flux did not have a defined direct cause because of the need to better understand and define what type of microbial activity was occurring to affect the organic acid levels. A change in the microbial population due to changes and range of temperature was possible; therefore, samples where taken at different times aligning to the four seasons to account for range of naturally occurring temperatures.

III. Methodology

Overview

The identification of the dominant species of soil bacterium was done by taking core samples from three variable depths at four seasonal intervals of the year. Samples were taken from the treatment wetland located on Wright-Patterson Air Force Base (WPAFB) and control samples were taken from a natural wetland approximately 5 miles distant at Valle Greene Marsh. Each core sample was split in quarters to minimize contamination. Sub-samples were then taken for 16s rRNA PCR and sequence analysis. The treated wetlands on WPAFB are known to have bacteria in them that dechlorinate PCE and TCE to ethane. Once the DNA was sequenced, the analysis was performed to show alignment to known reductive dehalogenases. A summary of processes and what documentation was used for each step can be found in Appendix A. Below are the basic steps that were taken from core collection through gene amplification. Timeline estimates and calculations are for the processing of one core, and the steps were performed each time for each core. See Figure 1 for an overview of the time line for one core. Figure 1 shows the entire process broken down into three main stages. The first stage contains the actual core extraction from the wetland and the removal of core from the aluminum tube that was used to extract it. Stage I is estimated to take between three to four days. Stage II in Figure 1 is the lab stage where the actual DNA extraction, cloning and sequencing occurs. This portion of the process is estimated to take four to five weeks. The last and final stage of the study in Figure 1 is stage III and embodies the statistical analysis of the study. Stage III is estimated to take a month to complete.

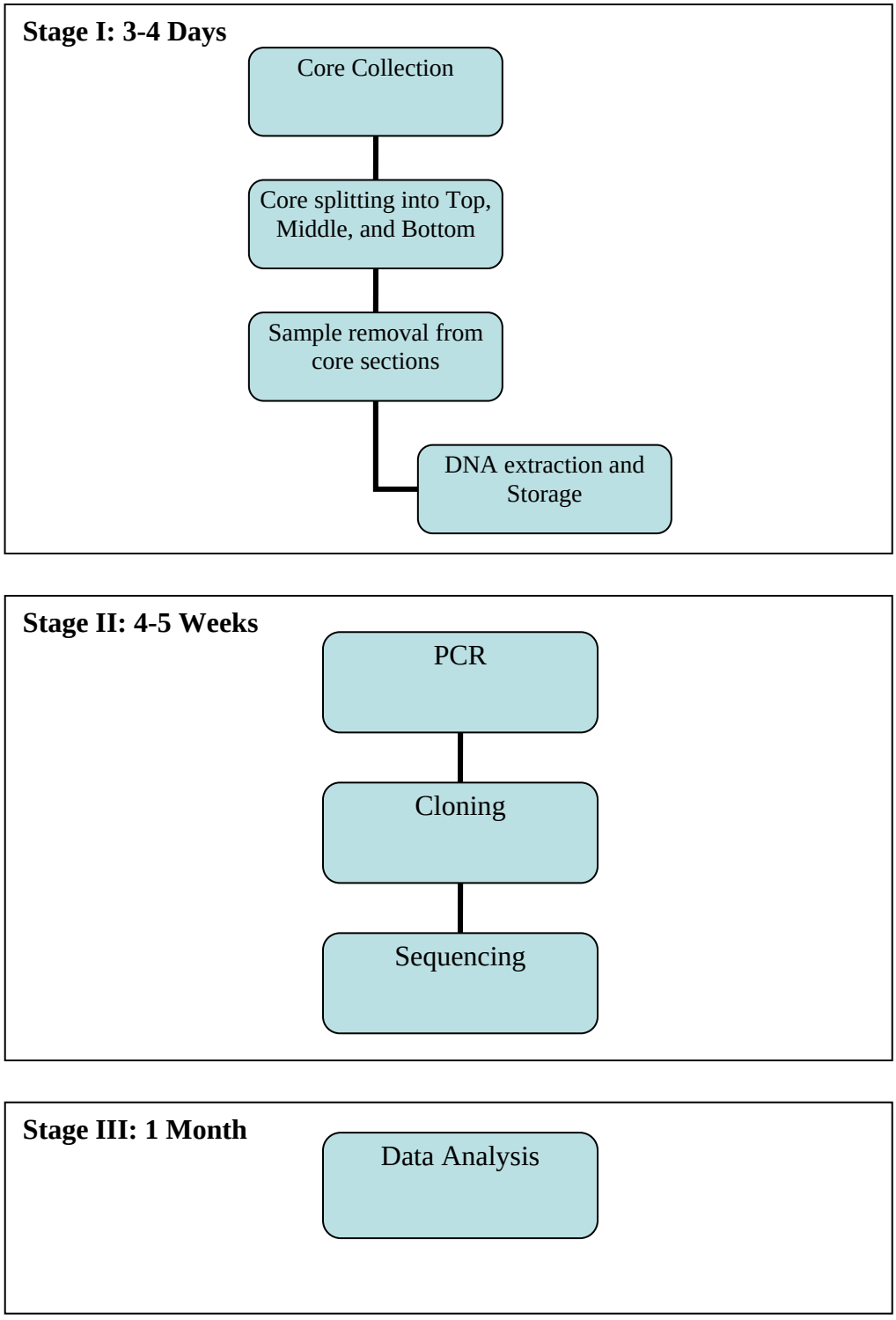


Figure 1. Overview of Timeline for Core Sample and Analysis

CORE COLLECTION

Extraction of Soil Samples

There were two sites chosen in order to extract the required soil samples. The control site at Valle Greene North, is a 12-acre site located east of I-675 and north of Dayton-Yellow Springs Road in Fairborn, Greene County, Ohio. The contaminated site is located on Wright Patterson Air Force Base (WPAFB), Dayton, Ohio and was constructed by researchers at the Air Force Institute of Technology and Wright State University to examine whether a wetland system could degrade chlorinated ethenes. This system is an upward flow system measuring 18.3 meters wide by 33.5 meters long. This wetland has a total volume of approximately 7.5×10^5 L and contains 1.6×10^7 grams of soil (dry weight). This wetland has three distinct 45 cm layers (Figure 2). The deepest layer is a former hydric soil, linwood muck, into which was placed methanogenic seed in the form of a mixture of sludge and muck from a natural wetland. The middle layer is a mixture of yard waste compost and soil in a 1:8 ratio. The purpose of the compost is to provide a slow release of substrates for methanogenesis. The top layer is soil only. Water is pumped into the bottom of the wetland and flows upward through the sediments (Kovacic, 2003).

The upward flow design of this wetland mimics a type of natural upflow wetland called a fen. Fens are typically found where groundwater discharges to the surface (Mitsch and Gosselink, 1993). They are characterized by year round saturation and accumulation of un-decomposed plant matter. Fens are typically nutrient poor due to the constant flushing effect of groundwater inflow and the lack of degradation of organic

material due to anoxic conditions in the sediments. Fens often support plant species adapted especially for limited nutrient conditions and full saturation (Kovacic, 2003).

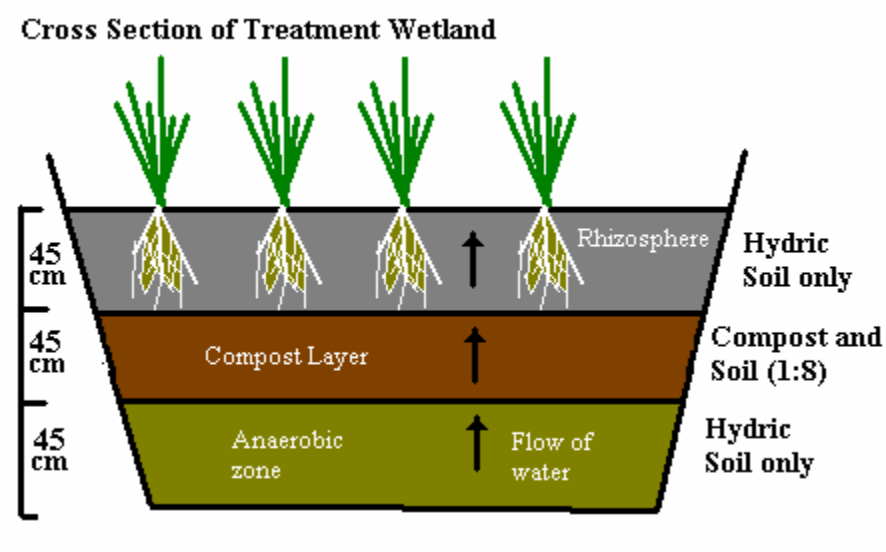


Figure 2. General cross-section of treatment wetland for bioremediation of chlorinated solvents on Wright Patterson Air Force Base, Dayton, OH.

In this same environment at WPAFB wetland, it was found by Slusser (2001) that extensive anaerobic degradation of PCE from the microcosm occurs. This process and how it works in relation to micro-organisms and the surrounding plant life is the focus of this study. Another factor that helped to identify where the core samples would be taken was the professional recommendation of Dr. Amon at Wright State University (WSU). He pointed out that the *Carex camosa* species of marshland plants were found to help degrade PCE in laboratory experiments. Because of Dr. Amon's recommendations, the first core samples were taken randomly from an area that had a comparatively high concentration of *Carex camosa* species of plants. This translated into a one meter square area of ground that had at least eight *Carex camosa* plants. Once this area was identified, all future samples were taken randomly from this same square meter of soil. There is a

single area identified at each site, both the Valle Greene control site and the WPAFB wet land site from which random samples were taken. Each site had four core samples taken every three months with the first occurring in January (the winter season). Each core sample was taken with three separate four inch diameter aluminum tubes. Each set of three tubes correspond to three different depths extracted from the same hole. The overall depth of the entire core sample averaged 1.35 meters, with each of the three tube lengths extracting approximately 45 cm section of the overall 1.35 meter depth. The different lengths were used to separate the three individual layers of the WPAFB wetland and to allow for easier insertion of the aluminum tube and extraction of soil. The layers can be seen in Figure 2 and their description is at the beginning of this section. The seasonal division of the four samples is performed because of the observance of the above ground seasonal changes to the plant life.

Eight core samples were taken. Each time a core was taken, it would consist of three separate aluminum tubes of varying length. The different lengths corresponded to the three depths or sections that were to be extracted with the top most section being labeled as section one. Once a location was selected, a rubber plunger was placed in the tube base with a nylon rope attached to the end of the stopper facing the inside of the tube which can be seen in Figure 3.

Figure 3. Aluminum tube used for soil extraction showing rubber plunger insert

The plunger was meant to prevent water from entering the tube as it was placed in the soil. The plunger also acted as a vacuum seal once the tube was extracted from the hole. Once the tube was in position, the nylon rope was looped around a medal rod at the top of the tube and strung through a hole that was drilled in the side of the tube.

Figure 4. Feeding plunger rope through wall of aluminum tube

The nylon rope was secured to a stationary point to help hold the plunger at the tube's starting position, which helped prevent soil compression as the tube was driven

into the soil. Once the rope was secure, a metal header was placed over the top of the aluminum tube to disperse the impact from the sledgehammer used to drive the tube to the desired depth.

Figure 5. Driving aluminum tube into wetland soil

Figure 6. Pulley on tripod mount used to extract aluminum tube from wetland soil

A tripod with a pulley system was used to extract the core from the soil. The tripod also acted as a stationary point for the rope attachment. Once the tube was at the desired depth, the metal rod used to guide the nylon rope through the hole in the side of the tube was used to attach the tripod pulley system. The extraction of the core was done slowly to prevent the soil from slipping out of the tube as it was pulled to the surface. At frequent intervals, the extraction process would halt and the tube was rotated back and forth to loosen its grip to the surrounding soil. During the extraction of the tube, constant tension was kept on the nylon rope attached to the plunger, which helped to prevent the soil from slipping out of the tube during extraction.

Once the tubes were extracted, they were sealed with plastic and marked with the location, date, and depth of extraction. This method was an efficient way of extracting soil samples from a saturated wetland and performed consistently, once the process was mastered. There was a small amount of soil compression, but this was minimized by slowing the process of driving the tube in the soil.

CORE SPLITTING

Transfer soil samples to tubes

Once the soil was extracted from the ground, the aluminum tubes were taken to the Wright State University (WSU) green house to be cut open lengthwise. The tubes were cut using a table saw with a metal cutting blade set to cut at a depth just deep enough to penetrate the aluminum wall of the tube. Two cuts were made on each tube approximately one hundred and eighty degrees from each other on the circumference of the tubes exterior. Once the tube was split, one half was removed and the soil sample was extracted from the reaming half, wrapped in plastic and labeled. Once the aluminum tube was removed, the soil samples were then placed in a refrigerator at approximately 4^0C until the DNA extraction process was performed.

Figure 7. Prying apart aluminum tube sections to extract soil

Figure 8. Exposed soil sample from separated aluminum tube halves

SAMPLE REMOVAL

Before the DNA was extracted from the soil, each section was split in half and one half was re-wrapped in plastic and placed in cold storage. The other half was split again and soil was extracted and placed into sterile tubes. This process was done in a laminar flow box.

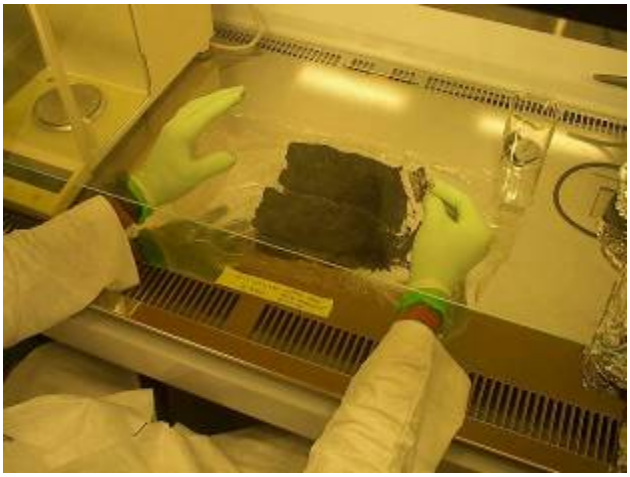


Figure 9. Dissection and extraction of soil from center of core sample

Each time the core was split; precautions were made to insure contamination was minimized. Once the core half was split, samples were taken along the entire length of

the middle of the split half. Approximately twenty-five grams of the soil sample were placed into Vulcan tubes. These tubes were then stored at -4º C until they were ready for DNA extraction.

DNA EXTRACTION FROM SOIL

DNA was extracted from the soil using the MO BIO Laboratories, Inc. PowerSoil DNA Isolation Kit, Catalog number 12888. Appendix B covers the step-by-step process used for the extraction and purification of the DNA. The soil samples were taken from the 10 gram samples extracted from each core section. All procedures were done in a laminar flow box. Gloves were washed with bleach solution any time they came out of the box and new gloves were put on between samples from different core sections. Two buffer negative controls were included with each extraction.

POLYMERASE CHAIN REACTION

Amplification of DNA using PCR and Electrophoresis validation

The amplification process was performed using the Qiangen HotStarTaq Master Mix kit and the primers used for the purification process were bacteria specific 16S rRNA sequence PCR primer code E8F (Sequence 5'-3' AGAGTTTGATCCTGGCTCAG) and primer code E533R (Sequence 5'-3' TIACCGIIICTICTGGCAC). Relatively consistent concentrations of DNA were produced by the extraction process which allowed for the standard ratio of biochemical compounds. A standard range of between 3-5 micro liters of template were used in the PCR. As per the QIAGEN instructions, there was a 15 minute initial activation step

before each PCR at 95ºC that was needed to activate the polymerase. The standard denaturation temperature was 94ºC and the annealing temperature was 46º C. The denaturation, annealing and extension steps each lasted 1 minute and occurred consecutively to form a three step cycle. The three step cycling was repeated with 30 cycles with a final extension for 10 minutes at 72ºC. Specific volumes and identification numbers with dates can be found in Appendix C along with results of the electrophoresis.

Once the DNA template was amplified through the PCR, the new amplified template was validated using electrophoresis. The first step required a Tris-acetic acid-disodium EDTA (TAE) solution, used as the base for making a 1% Agarose Gel for the gel bed and also used for the running buffer. In both the bed and buffer solution, ethidium bromide was added at 0.5 µg/ml. This increased the clarity of the visual results gained from the electrophoresis. Details for the processes are described in the Fisher Scientific horizontal electrophoresis systems (HES) manual, revision 1/2003. Two HES models were used in this study, FB-SB-710 for running between 1-19 samples, and FB-SBR-1316 for running 20- 38 samples. Configurations provided up to twenty or forty wells respectively, but one well per row was needed for a 1kb DNA ladder, the known scale for each run. The manual gives the step-by-step procedure for this process. The gel thickness used throughout this study was 0,50 cm thickness for the gel bed. A summary of the standard operating procedures is in Appendix D. Each gel used 3 µl of PCR sample mixed with 17 µl distilled water and 4 µl 6X buffer. The gel loading buffer was initial derived from the Invitrogen 10X BlueJuice Gel loading buffer catalog no. 10816-015. The specification sheet can be found in Appendix E. This was diluted into a

6X buffer solution for smaller sample sizes. The final solution of loading sample was 24 micro liters for each PCR and was then pipette into individual wells of the loading gel.

Electrophoresis Gels were run for 30 min. at 120 volts DC. Finished gels were recorded with an ultraviolet imaging system and the results were logged by date, slide number, and lane in Appendix C. Completed gels were viewed using the Kodac Gel Logic 200 Imaging System. Over 370 individually PCRs were run to acquire the 48 samples used for the next step in cloning.

CLONING

TOPO Cloning Reaction

Once two successful PCRs were run and validated from a single DNA template, the two PCRs were combined and labeled with a new serial number. The next step in defining the dominant species was the cloning and separation of individual DNA sequences using Invitrogen TOPO TA Cloning Kits, Version R with the PCR 2.1-TOPO with One Shot Cells of TOP10 vector. The chemically competent *E. coli* reactions were followed with the following details for setting up the TOPO cloning reaction. Table 2 shows the volumes used for this portion of the reaction.

Table 2. TOPO TA Cloning™ Reaction Reagent Volumes

Reagent	Chemically Competent *E. coli* Reagent Volume (µl)
Fresh PCR Pool Product	1
Salt Solution	1
Sterile Water	3
TOPO Vector	1
Final Volume	6

The PCR product referred to in Table 2 is taken from the combined PCR products that were from the previous procedure. The PCR pools and which PCR products that make up each pool can be found in the PCR Pool Log in Appendix F.

The next step in the cloning process, the "Transforming One Shot," followed the procedures in the Invitrogen life technologies TOPO TA Cloning Instruction Manual, Version R, 8 April 2004 (Appendix G, pages 5, 9-10). This aseptic process was performed in a laminar flow box.

Once the cloning reaction was complete, it was added to the vial of One Shot Chemically Competent *E. coli* and gently mixed. The vial was incubated for 30 minutes and then heat-shocked at 42ºC for 30 seconds. The next step added 250 µl of "S.O.C." at room temperature to the vial. Once the S.O.C. was added, the vial was capped and horizontally shaken at 200 rpm at 37ºC for 60 minutes. The samples were taped to the holding tray in the New Brunswick Scientific Co. Classic Bench top Incubator Shaker Model C-24KC.

Once the transformation was complete, the vials' contents were spread onto prewarmed Luria-Bertani (LB) plates. Kanamycin was the antibiotic used for this study.. Details are shown in Appendix G. Disposable sterile cell spreaders were used to apply the transformation solution to the LB plates.

Blank control plates were done for each set of plates. For the blank plate, a sterile spreading stick would spread across the surface of the hardened LB agar identical to the process followed for spreading the transformation solution. The control plates were incubated with the inoculated plates for 16 hours at 37ºC.

No blank plates had visible colonies after 16 hours. Each vial of transformation solution yielded three plates.. After incubation, plates were separated and prepped for colony selection. Figure 10 shows a typical plate with colonies evenly spread.

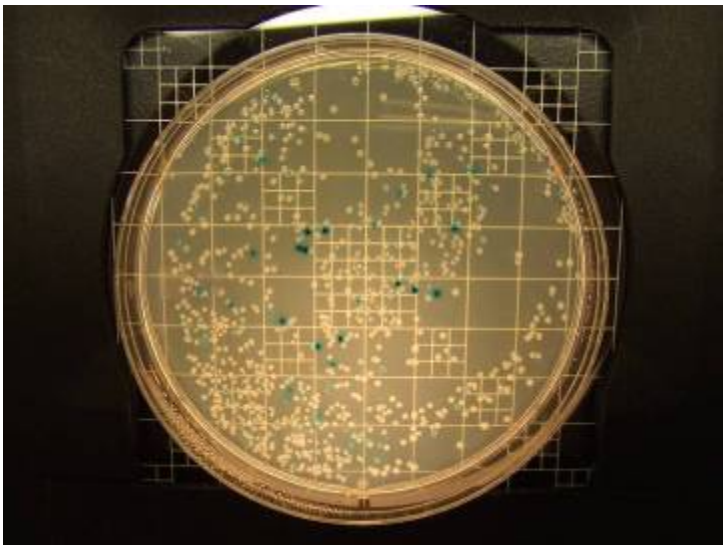


Figure 10. Cloned Competent Cells with Isolated DNA segments on LB medium containing 50µg/ml kanamycin and 1% Tryptone (Dark colonies are Vector only Clones)

The selection process requires the visual inspection of each plate and subsequent selecting of valid colonies. Valid colonies are those that are of average size and separated enough from other colonies to allow for an isolated extraction of the colony. Blue or dark colonies are not desirable, because they are indications of *E. coli* that have not successfully taken up the DNA of the piece desired from the extraction process. The blue colonies are referred to as a vector only reaction. The *E. coli* contains a plasmid, but the plasmid does not have any of the inserted DNA. The purpose for the colony selection is to isolate a single DNA sequence and then inoculate a glass test-tube filled with 2ml of LB medium. The tube is then incubated for 16 hours at 37ºC.

The plates that were control blanks were also processed along with the experimental samples. For the control samples, the extraction tool was rubbed across the

surface of the blank LB agar surface and then placed into the 2ml of LB marked as a blank control sample. This validated that the colonies on the other plates are due to the transformation solution and not an organism that was added from faulty process quality control.

Colony selection was done in a laminar flow box. Ten colonies were extracted from each plate with each extraction being placed in a separate test-tube. Once a tube was inoculated with an extracted sample, the tube was labeled and prepared for incubation. The labeling identified the incubation tube ID to the plate it came from and is summarized on the Incubation Tube ID table in appendix H.

Plasmid DNA Purification and Isolation

Plasmid isolation was performed using the QIAGEN Plasmid Isolation Kit QIAprep Spin Miniprep kit (250), Cat. No. 27106. This kit was chosen because of its elimination of the need for loose resins or slurries. The process used followed the protocol called out in the QIAGEN QIAperp Miniprep Handbook, second edition, June 2005 (22-23). The protocol is a 10 step process that finished with 50µl of buffer filled with plasmid DNA.

Digestion/Validation

After plasmid isolation, a validation was performed on the first 160 samples referred to digestion. The digestion process uses enzymes called restriction endonucleases. The kit used for the digestion process was the Promega-Usage Information Sheet (EcoR I) Catalog # R6011 (Appendix I). The following table indicates

the amounts of reagents used for the digestion. The regents were added in the order they appear in the table. The digestion period was for 2 hours.

Table 3. Promega Restriction Enzyme Digest (EcoRI) Reagent Volumes

Regents	**Volumes (µl)**
Sterile, deionized water	12.3
RE 10X Buffer (H)	2.0
Acetylated BSA (10µg/µl)	0.2
Cloned DNA	5.0
Mix by pipetting	
Restriction Enzyme (ER1)	0.5
Final Volume	20.0

These enzymes are used to locate specific DNA segments that have been joined to infectious DNA and delivered to microorganisms. This process is also referred to as cutting and leaves smaller segments of DNA that are cut at specific points referred to as restriction sites. The validation step confirms that the preceding process was successful by confirming the fact that these restriction sites exist and that they are uniform. The uniformity is confirmed by performing electrophoresis on the digested samples which measures the size of each segment. A successfully cloned DNA segment, once digested, should break into two segments of approximately 1kbp and 500bp with the 500 bp being the gene sequence extracted from the soil microorganisms and the 1kbp being the plasmid vector used to infect the *E. coli* microbe. The first 160 digestions had a 100% success rate. Because of this, the digestions validation was waved on the remaining samples and they went directly to the DNA sequencing preparation steps.

SEQUENCING

Measurement of DNA template concentrations

Since the sequencing process has a minimum and maximum concentration for optimal outcome, the DNA template concentrations were measured using a Nano-drop. The values for each sample can be found in Appendix J. The amount of DNA needed was chosen based on the table for estimating the dsDNA concentration in the Beckman Coulter-GenomeLab Methods Development Kit Dye Terminator Cycle Sequencing (p1-4), Spec Sheet 608019-AR (March 2005) found in Appendix K. The concentration 260 ng for 100 fmol was used because of the preliminary success results of Major Ethan Bishop while sequencing similar samples for his masters thesis.

Preparation of DNA sequencing reaction

Once the cloned and isolated DNA segments had been validated, the next process step was to prepare the samples for the Beckman Coulter sequencer. The following procedures are prescribed in the Beckman Coulter-GenomeLab Methods Development Kit Dye Terminator Cycle Sequencing (p1-4), Spec Sheet 608019-AR (March 2005), using dITP chemistry. This first step involved the preparation of the premix with all the reagents for attaching the fluorescent markers to the specific nucleotides. This was done for the dITP chemistry solutions. The amount of water and DNA template to be added to each sequencing sample are found in sequencing tables for each sequence and are located in Appendixes L-Q. Once the water and DNA template were mixed, the samples were pre-heated at 96ºC for 1 minute to help break apart the DNA to allow for a higher opportunity for the polymerase, dNTP dyes, and ddNTP joining to the nucleotides in the

segments. Once the DNA templates were mixed with the dyeing regents and thermal cycled with the dITP cycling program, the samples then went through an ethanol precipitation step.

Ethanol Precipitation

The ethanol precipitation was done in a cold room with a constant temperature of 4ºC. This was very difficult process in that many times the DNA precipitate could not be seen in the tube. This section followed the Beckman Coulter-GenomeLab Methods Development Kit Dye Terminator Cycle Sequencing (p1-4), Spec Sheet 608019-AR (March 2005) used in the proceeding step.

Compile Sequencing Data into FASTA format

Once the sequencer had analyzed the samples it provided the results in FASTA format, which is a standard protocol developed to query genomic databases for like sequences. Each sample generated its own FASTA file and each file needed to be batched with a master file that could be uploaded into the BLAST GenBank on the NCBI website. Each FASTA file can be seen in Appendix R-W and is organized by sample number.

Query Nucleotide Data into BLAST GenBank

Once the FASTA files were uploaded into the BLAST query engine, the outputs had to be verified and sorted. Each sample can generate hundreds of hits and they are ranked by their E-Value and then their bit score. The E-value is the number of times the

database match may have occurred by chance. The lower the E-value, the more similar the hit and query is, and the greater the confidence in the hit. The bit-score measures the statistical significance of an alignment with the higher number being better. The results were compiled by taking the top hit of each query. This information is summarized and tied to the core it came from in the AFIT/WSU Event Log in Appendix J. The definitions for each of the number one top hit for each sample referenced in the Version Definition List in Appendix X.

Assumptions/Limitations

- L This study will not be able to analyze the surrounding soil at time of core extractions
- L Once a sample of soil is removed, that particular soil cannot be recycled back in eco system.
- Soil samples are homogeneous and representative of entire samples.
- A There are significant differences in the soil microbial community from different core samples taken in the same general area.
- Soil with higher root content or different characteristics are all treated and weighed the same.
- The microbial community will not vary significantly from soil taken close to roots and that taken from soil outside the XXX region.

IV. Results and Discussion

Sampling

The results were summarized in a histogram and a scatter plot that both showed the sampling bias and the dispersion of results taken. These graphical representations help support the findings that thousands of sampling events are required before any reliable predictions can be made about the community total population (Curtis, 2004). The histogram in Figure 11 shows the data collected for the complete set of samples taken from both the control and treatment. The histogram demonstrates the phylo-type occurrences are skewed and not representative of a normal distribution. The Figure 11 scatter plot demonstrates that there is no strong relationship to the phylo-types and the locations they were extracted from. The scatter gram is truncated and sorted to show only those samples that had two or more of same phylo-type.

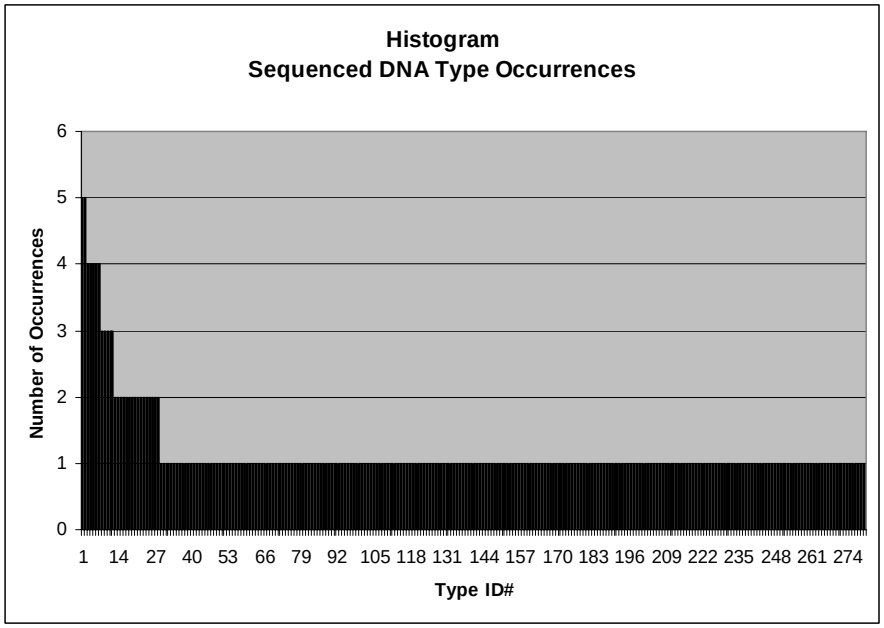
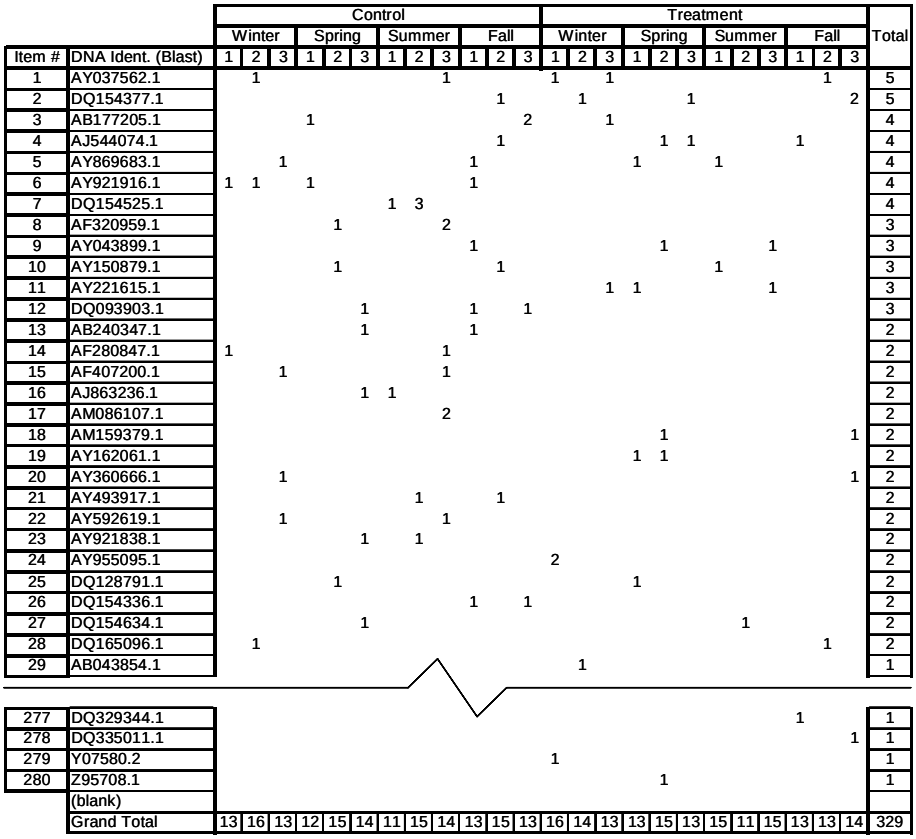


Figure 11. Sequenced DNA Histogram demonstrating the comparatively large occurrences of unique phylo-types



		Control												Treatment												Total
		Winter			Spring			Summer			Fall			Winter			Spring			Summer			Fall			
Item #	DNA Ident. (Blast)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	
1	AY037562.1		1							1				1		1								1		5
2	DQ154377.1											1			1				1						2	5
3	AB177205.1				1								2			1										4
4	AJ544074.1											1						1	1				1			4
5	AY869683.1			1							1						1			1						4
6	AY921916.1	1	1		1						1															4
7	DQ154525.1							1	3																	4
8	AF320959.1					1				2																3
9	AY043899.1										1							1				1				3
10	AY150879.1					1						1								1						3
11	AY221615.1														1	1						1				3
12	DQ093903.1						1				1		1													3
13	AB240347.1						1				1															2
14	AF280847.1	1								1																2
15	AF407200.1			1						1																2
16	AJ863236.1						1	1																		2
17	AM086107.1									2																2
18	AM159379.1																	1							1	2
19	AY162061.1																1	1								2
20	AY360666.1			1																					1	2
21	AY493917.1								1			1														2
22	AY592619.1			1						1																2
23	AY921838.1						1		1																	2
24	AY955095.1													2												2
25	DQ128791.1					1											1									2
26	DQ154336.1										1		1													2
27	DQ154634.1						1														1					2
28	DQ165096.1		1																					1		2
29	AB043854.1														1											1
277	DQ329344.1																						1			1
278	DQ335011.1																								1	1
279	Y07580.2													1												1
280	Z95708.1																	1								1
	(blank)																									
	Grand Total	13	16	13	12	15	14	11	15	14	13	15	13	16	14	13	13	15	13	15	11	15	13	13	14	329

Figure 12. Truncated scatter plot of phylo-type occurrences relating to extraction locations with highest occurring phylo-type appearing as Item #1

The first indication that the process demonstrated the amplified products were heterogeneous was during the validation electrophoresis of the restriction digestion products. Figure 13 shows a slide taken of an electrophoresis performed on DNA that has been cloned and isolated.

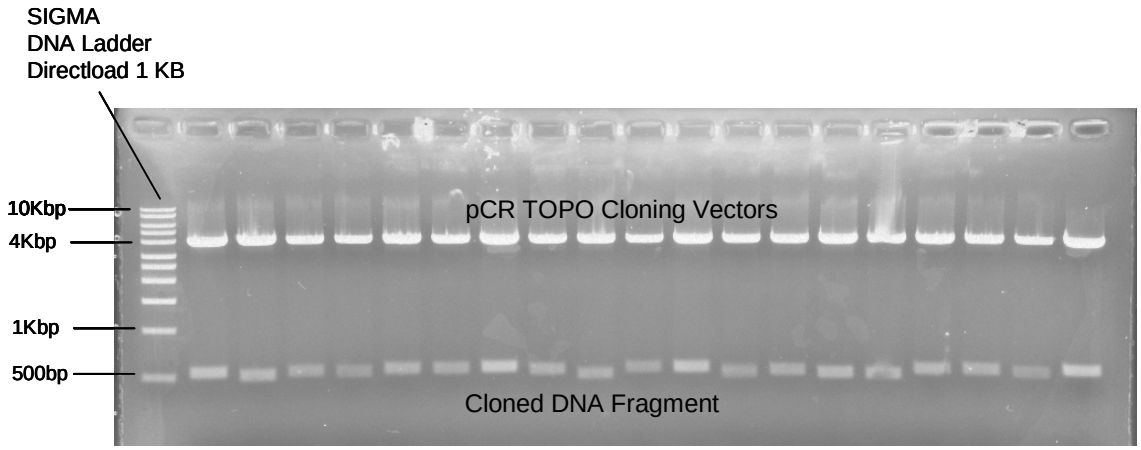


Figure 13. Gel electrophoresis used to separate cloned DNA segments that have been separated from their vectors through restriction digestion process, used as a process validation before samples are prepped for sequencing

The inserts were then removed from the Plasmid DNA. The top row is the larger fragment of DNA of the Plasmid and is of a uniform length of 3.9 kB. The bottom row is the DNA inserts that were cut from the plasmid DNA and have a slight variation in their length. The spec sheet on the plasmid DNA can be found in Appendix G and shows that the insert fragment is from 1-547 bp in length. The ladder used is from Sigma and its spec sheet can be found in Appendix Y. Some key values are shown in the figure to help show the sizes of DNA that was isolated. The output from the sequencer can be found in Appendix R-W and indicates the size of the isolated DNA fragments that were isolated from the wetlands. Variation in size can originate from several factors because the process of polymerase chain reaction is one of random pairing. The resilience of the DNA fragment being amplified can allow DNA to break apart and primers can attach at staggered positions resulting in variations in segment length. Another reason for variation results from the abundance of tails that tend to be rich in identical nucleotides that are not always conducive to primers attaching at the ends. Lastly, variations in

length can be due to different sequences occurring because of different organisms having different genetic sequences. The restriction endonucleases are enzymes that act as scissors to cut DNA at restriction sites. Certain sections of DNA from one organism to another is referred to as conserved, meaning there is little change from one organism to another. These enzymes can reliably cut DNA from different organisms at close to the conserved positions. Once a segment is cut, primers are used to attach to the segments and act as a starting and ending points. These points bracket the segment that will be duplicated (Drlica, 2004). An analysis of the output lengths from the sequencer shows a fairly consistent range of output lengths in the 500bp range. Table 4 shows the Mean, Median, and Standard Deviation of the cloned insert lengths.

Table 4. Statistics on the cloned insert lengths after being sequenced

Mean =	467.4
Median =	499.0
SD =	99.2
Range =	74 - 625

Figure 14 is a histogram of the sequences output lengths, which demonstrates the grouping of sizes at the expected values.

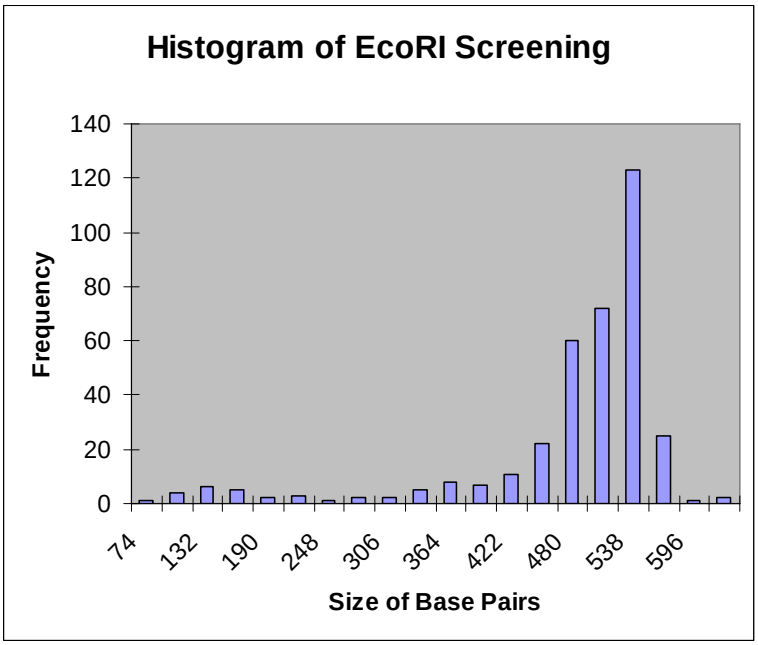


Figure 14. Histogram of cloned insert lengths after being sequenced

The output length is actually skewed to below 500bp length. This could be the result of restriction enzymes cutting segments in places that had the same sequences of nucleotides somewhere in the middle portion of the desired DNA segment.

Population Comparison by Sample

The original goal of this thesis was to identify dominant microbial species based on DNA analysis and make observations of trends related to season, soil depth, and treatment/control wetlands. It was found that a microbial ecology exists with such large diversity, that this thesis was unable to gain a statistically viable representation of the population. The original questions posed of this thesis were unable to be answered; although some broad observations were made that can be used to direct future research.

The samples identified were evenly distributed over both the control wetland at Valle Greene and the treatment wetland at Wright-Patterson Air Force Base, OH. Unless

otherwise stated, all probabilities are calculated with the assumption that sample types have an equal probability of being identified from the population, samples are homogeneous, and samples represent the actual population. Three hundred and twenty-nine (329) samples were successfully identified. Of the 329 successful samples, one hundred and sixty-four (164) samples identified microbes for the control wetland and one hundred and sixty-five (165) samples identified microbes for the treatment wetland. There were a total of eight cores taken with three sections per core for a total of twenty-four sections. The mean of the number of successful samples for the twenty (24) sections was fourteen (14) and the median was fourteen (14). This shows an unbiased distribution of samples. This can be seen in Table 5 and is grouped first by wetland type, then by core ID and finally by section.

Table 5. Counts of successfully identified samples by wetland and core section

	Valle Greene (Control)												
Core ID	1			5			7			9			Total
Section ID	1	2	3	1	2	3	1	2	3	1	2	3	
Sample Count	13	16	13	12	15	14	11	15	14	13	15	13	164
Mean of Samples	14.0			13.7			13.3			13.7			

	WPAFB (Treatment)												
Core ID	2			4			6			8			Total
Section ID	1	2	3	1	2	3	1	2	3	1	2	3	
Sample Count	16	14	13	13	15	13	15	11	15	13	13	14	165
Mean of Samples	14.3			13.7			13.7			13.3			

It was originally hypothesized that a set of ten (10) samples per section would give a good representation for the diversity in the wetlands. The resulting data shows there was such a large diversity, even with an average of fourteen (14) samples per section, a statistical identification of the population was not possible. Of three hundred

and twenty-nine (329) samples taken, two hundred and fifty-two (252) samples taken were unique. Of the seventy-seven (77) samples that occurred more than once, each only had an average reoccurrence of 2.75. Table 6 graphically summarizes the sample types and shows which wetlands they occurred in. The location column contains those samples found in the control wetland under the column heading "0" and the samples found in the treatment wetland under the column heading "1". The table is divided into three parts along the left hand side to represent those types that occur in which wetland. Part I of the table shows those types that occur in both wetlands, Part II shows those types that occur in only the control wetland and Part III show only those types that occur in the treatment wetland.

Table 6. Summary of multiple occurring phylo-types by wetland

	DNA Ident. (Blast)	Location		Grand Total
		0	1	
Part I	AY037562	2	3	5
	DQ154377	1	4	5
	AB177205	3	1	4
	AJ544074	1	3	4
	AY869683	2	2	4
	AY043899	1	2	3
	AY150879	2	1	3
	AY360666	1	1	2
	DQ128791	1	1	2
	DQ154634	1	1	2
	DQ165096	1	1	2
Part II (VG)	AY921916	4		4
	DQ154525	4		4
	AF320959	3		3
	DQ093903	3		3
	AB240347	2		2
	AF280847	2		2
	AF407200	2		2
	AJ863236	2		2
	AM086107	2		2
	AY493917	2		2
	AY592619	2		2
	AY921838	2		2
	DQ154336	2		2
Part III (WPAFB)	AY221615		3	3
	AM159379		2	2
	AY162061		2	2
	AY955095		2	2
			Total =	77.00
			Mean =	2.75
			Median =	2.00

For those samples that only occurred once, one hundred and thirty-five (135) occurred in the control wetland and one hundred and seventeen (117) occurred in the treatment wetland. With the current population, the probability of finding a unique microbe in the control wetland is 82.3% (135/164) compared to a probability of finding a unique microbe in the treatment wetland is 70.9% (117/165). This could lead to the observation that the diversity is greater in the control wetland. However, if the total number of different microbes that were found in each wetland is compared regardless of whether it was unique or reoccurring, a different observation is made. There were a total of two hundred and eighty (280) different sequencing results identified between the two wetlands with eleven types that occur in both. One hundred and forty (140) different types were identified for the control wetland and one hundred and fifty-one (151) different types were identified for the treatment wetland. The number of samples for each wetland was considered equal so the proportions relating the two wetlands can be assumed equal proportional. The ratio of different microbes found in the treatment wetland compared to the control is 1.08. This gives a slight almost insignificant bias for having a greater diversity in the treatment wetland.

If we employ the S_{Chao1} (Chao 1984, 1987) abundance-based diversity and C (Chao, 2004) coverage proportion estimator method to compare the diversity between the treatment and control wetland we come up with the following values listed in Tables 7 and 8.

Table 7. Abundance Based Diversity (ABD) calculation values and results for the Valle Greene wetland samples

Valle Greene (Chao)	
S_{obs} =	140
F_1 =	123
F_2 =	12
N_{rare} =	140
C_{ACE} =	0.12
S_{Chao1} =	717.518

Valle Greene (Good)	
n_1 =	123
N =	140
C =	0.12

Table 8. Abundance Based Diversity (ABD) calculation values and results for the Wright-Patterson Air Force Base wetland samples

WPAFB (Chao)	
S_{obs} =	151
F_1 =	142
F_2 =	5
N_{rare} =	151
C_{ACE} =	0.06
S_{Chao1} =	1821.5

WPAFB (Good)	
n_1 =	142
N =	151
C =	0.06

Tables 7 and 8 show the values used to calculate the Abundance Based Diversity (ABD) based on the phylotypes found for the Valle Greene (control wetland) and the Wright-Patterson Air Force Base (treatment wetland). The calculations indicate that the treatment wetland has a calculated 2.5 times greater ABD then the control wetland when the Chao

method is used. If the coverage (Good) is compared, the treatment wetland is estimated to have over twice (2.2) as many phylotypes as the control wetland. It seems that either method indicates the same comparison that the treatment wetland potentially has a greater abundance of phylotypes then the non-contaminated control wetland. Both the coverage estimators from Chao and Good yielded the same results. This was primarily due to the fact that there was negligible phylotypes that occurred more then twice but less then ten times.

V. Conclusions and Recommendations

Synopsis

The purpose of this study was to identify the dominant microbes through genomic analysis and identification. It was shown in prior studies that organic acids, which are indicators of microbial activity, were found roughly about 27 to 45 inches below the surface of the treatment wetland and close to the surface to about 9 inches below the surface (Kovacic, 2003). The microbes of interest were those that live in a treatment wetland that has a known diverse distribution of geochemical processes. Samples for the study were taken at three different stratus of an upward flow constructed wetland located at Wright-Patterson Air Force Base. The initial requirements of the study were to take one core sample for each calendar season and divide the core into three sections. Each section was then to have ten genomic identifiers gained through the 16s rRNA PCR and sequence analysis. The analysis process was used to determine species dominance and any statistical trends between the different strata of the wetland or seasonal trends. Parallel samples were taken from a local wetland that was assumed to be free of hydrocarbons as a control to compare results.

Findings from both wetlands indicated an extremely diverse microbial community. The sample size originally chosen was not large enough to represent the population; nor were any statistically significant trends able to be identified. The data did seem to suggest that the diversity in the treatment wetland was greater, but the margin of reliability could not be determined and the difference in diversity could in fact be within any limits of acceptable deviation from the expected values.

Recommendations

The primary limitations of the inferences that could be made in this study is the timeframe in which it was conducted and the sample size taken. The process of extracting soil, isolating DNA from the soil, preparing the DNA for sequencing, sequencing and analysis the data are not only time consuming, but very technical and necessitates highly trained and specialized personnel. It would seem practical for future attempts to limit the scope to a single season or depth and increase the number of samples for that limited scope. A set of control samples offered minimal benefit to the study and should only be done when attempting to isolate specific difference that may exists between a contaminated treatment wetland and contaminant free natural wetland.

A future study could look at a single core sample divided into the three sections of the treatment wetland at WPAFB and take several hundred samples of each section in order to gain a statistically valid representation of the population. The samples should be taken in the winter months to correspond with prior studies that report on the organic acid level found in the treatment wetland. It would also be interesting to explore the use of other primers that would lead to more specific genomic sequences.

Conclusion

The results indicate that there is a very diverse microbial community throughout the strata of the treatment and control wetlands of which magnitude was unexpected. The results suggest that the diversity may be greater in the treatment wetland than that of the control wetland. The sampling size was not large enough to establish a statistically viable representation of the microbial population at any site or depth.

Appendix A: Wetlands Study Project Steps Documentation

1. **Take core samples from treatment wetland and control wetland.**
2. **Transfer soil samples to tubes**
3. **Extract the DNA from soil**
    - **a.** MO BIO-Instruction Manual Powersoil DNA Isolation Kit
    - **b.** Catalog No. 12888-100 (p1-8)
4. **Amplification of DNA using PCR**
    - a. QIAGEN-Instruction (p16-17) - HotStarTaq Master Mix
5. **Electrophoresis to validate DNA**
    - a. Fisher Scientific-Owners Manual - "Horizontal Electrophoresis System"
    - b. Invitrogen - 10X BlueJuice Gel Loading Buffer
6. **Combine successful PCR into a pool**
7. **Set up TOPO Cloning Reaction**
    - a. TOPO TA Cloning kit pCR 2.1-TOPO-TOP10) 45-0641
    - b. Invitrogen-TOPO TA Cloning Instruction Manual (p5)
8. **Transforming One Shot TOP10 Competent Cells**
    - a. Invitrogen-TOPO TA Cloning Instruction Manual (p9-10)
        - i. This step is what produces the LB-Plates
9. **Analyzing Transformants**
    - a. Invitrogen-TOPO TA Cloning Instruction Manual (12)
        - i. This step produces overnight test tubes of LB with ecoli
10. **Plasmid DNA Purification**
    - a. QIAGEN-QIAprep Miniprep Handbook (p21-23)
        - i. QIAprep Spin Miniprep kit (250) Cat. No. 27106
        - ii. This step produces DNA template for Sequencing
11. **Restriction-Digestion**
    - a. Promega-Usage Information Sheet (EcoR I)
        - i. Catalog # R6011
12. **Electrophoresis to validate**
    - a. Fisher Scientific-Owners Manual - "Horizontal Electrophoresis System"
    - b. Invitrogen - 10X BlueJuice Gel Loading Buffer
13. **Preparation of DNA sequencing reaction**
    - a. Beckman Coulter-GenomeLab Methods Development Kit Dye Terminator Cycle Sequencing (p1-4)
        - i. Spec Sheet 608019-AR (March 2005)
        - ii. Using dITP Chemistry
    - b. Reaction prep and thermal cycling
    - c. Ethanol Precipitation
14. **Sequence DNA**
15. **Compile Sequencing Data into FASTA format**
    - a. Bioinformatics for Dummies (p50)
16. **Quary Nucleotide data into BLAST GenBank**
    - a. BLAST - Basic Local Alignment Search Tool
    - b. NCBI – National Center for Biotechnology Information
17. **Data Mine results**

PowerSoil™ DNA Isolation Kit

Catalog No.	Quantity
12888-50	50 Preps
12888-100	100 Preps

Instruction Manual

Introduction

The PowerSoil™ DNA Isolation Kit* is comprised of a novel and proprietary method for isolating genomic DNA from environmental samples. The kit is intended for use with environmental samples containing a high humic acid content including difficult soil types such as compost, sediment, and manure. Other more common soil types have also been used successfully with this kit. The isolated DNA has a high level of purity allowing for more successful PCR amplification of organisms from the sample. PCR analysis has been performed to detect a variety of organisms including bacteria (e.g. *Bacillus subtilis, Bacillus anthracis*), fungi (e.g. yeasts, molds), algae and Actinomycetes (e.g. *Streptomyces*).

The PowerSoil DNA Isolation Kit distinguishes itself from MO BIO's UltraClean™ Soil DNA Isolation Kit with a **NEW** humic substance/brown color removal procedure. This new procedure is effective at removing PCR inhibitors from even the most difficult soil types.

Environmental samples are added to a bead beating tube for rapid and thorough homogenization. Cell lysis occurs by mechanical and chemical methods. Total genomic DNA is captured on a silica membrane in a spin column format. DNA is then washed and eluted from the membrane. DNA is then ready for PCR analysis and other downstream applications.

This kit is for research purposes only. Not for diagnostic use.

***PATENT PENDING**

Version: 09142005

Technical Information: Toll free 1-800-606-6246, or 1-760-929-9911
Email: technical@mobio.com

Required Equipment:
Microcentrifuge (10,000 x *g*)
Pipettors (50 µl - 500 µl)
Vortex
Vortex Adapter (MO BIO Catalog # 13000-V1)

Kit Contents

	Kit Catalog # 12888-50		Kit Catalog # 12888-100	
Component	**Catalog #**	**Amount**	**Catalog #**	**Amount**
PowerBead Tubes (contain 750µl solution)	12888-50-PBT	50	12888-100-PBT	100
PowerSoil Solution C1	12888-50-1	3.3 ml	12888-100-1	6.6 ml
PowerSoil Solution C2	12888-50-2	14 ml	12888-100-2	28 ml
PowerSoil Solution C3	12888-50-3	11 ml	12888-100-3	22 ml
PowerSoil Solution C4	12888-50-4	72 ml	12888-100-4	144 ml
PowerSoil Solution C5	12888-50-5	30 ml	12888-100-5	2 x 30 ml
PowerSoil Solution C6	12888-50-6	6 ml	12888-100-6	12 ml
PowerSoil Spin Filters (units in 2 ml tubes)	12888-50-SF	50	12888-100-SF	100
PowerSoil 2 ml Collection Tubes	12888-50-T	200	12888-100-T	400

Kit Storage
Kit reagents and components should be stored at room temperature (15-30°C).

Precautions
Please wear gloves when using this product. Avoid all skin contact with kit reagents. In case of contact, wash thoroughly with water. Do not ingest. See Material Safety Data Sheets for emergency procedures in case of accidental ingestion or contact. All MSDS information is available upon request (760-929-9911) or at www.mobio.com. Reagents labeled flammable should be kept away from open flames and sparks.

WARNING: Solution C5 contains ethanol. It is flammable.

IMPORTANT NOTE FOR USE: Make sure the 2 ml PowerBead Tubes rotate freely in your centrifuge without rubbing.

Experienced User Protocol

Please wear gloves at all times

1. To the PowerBead Tubes provided, add 0.25 gm of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60µl of Solution C1 and invert several times or vortex briefly.
5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog No. 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).
   Note: Expect between 400 to 500µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600µl of supernatant to a clean 2 ml Collection Tube (provided).
11. Add 200µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750µl of supernatant into a clean 2 ml Collection Tube (provided).
14. Add 1200µl of Solution C4 to the supernatant and vortex for 5 seconds.
15. Load approximately 675µl onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675µl of supernatant to the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. **Note**: A total of three loads for each sample processed are required.
16. Add 500µl of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g*.
19. Carefully place Spin Filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.
20. Add 100µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog No. 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.
22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

    We recommend storing DNA frozen (-20° to -80°C). Solution C6 contains no EDTA. To concentrate the DNA see the Additional Information Section.

Thank you for choosing the PowerSoil DNA Isolation Kit.

Detailed Protocol

Please wear gloves at all times

1. To the PowerBead Tubes provided, add 0.25 gm of soil sample.
   After your sample has been loaded into the PowerBead Tube, the next step is a homogenization and lysis procedure. The PowerBead Tube contains a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation.

2. Gently vortex to mix.
   Gentle vortexing mixes the components in the PowerBead Tube and begins to disperse the sample in the PowerBead Solution.

3. **Check Solution C1**. If Solution C1 is precipitated, heat solution to 60°C until the precipitate has dissolved before use.
   Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will form a white precipitate in the bottle. Heating to 60°C will dissolve the SDS and will not harm the SDS or the other disruption agents. Solution C1 can be used while it is still warm.

4. Add 60µl of Solution C1 and invert several times or vortex briefly.

5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog No. 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

   Note: *The vortexing step is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from steps 1-4 and mechanical shaking introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with microbial cells will cause the cells to break open.*

   The MO BIO Vortex Adapter is designed to be a simple platform to facilitate keeping the tubes tightly attached to the vortex. It should be noted that although you can attach tubes with tape, often the tape becomes loose and not all tubes will shake evenly or efficiently. This may lead to inconsistent results or lower yields. Therefore, the use of the MO BIO Vortex Adapter is a highly recommended and cost effective way to obtain maximum DNA yields.

6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.

7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).

Note: *Expect between 400 to 500µl of supernatant at this step. The exact recovered volume depends on the absorbancy of your starting material and is not critical for the procedure to be effective. The supernatant may be dark in appearance and still contain some soil particles. The presence of carry over soil or a dark color in the mixture is expected in many soil types at this step. Subsequent steps in the protocol will remove both carry over soil and coloration of the mixture.*

8. Add 250µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
   Solution C2 contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g.*

10. Avoiding the pellet, transfer up to 600µl of supernatant to a clean 2 ml Collection Tube (provided).
    The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

11. Add 200µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.
    Solution C3 is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g.*

13. Transfer up to 750µl of supernatant to a clean 2 ml Collection Tube (provided).
    The pellet at this point contains additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.

14. Add 1.2ml of Solution C4 to the supernatant (be careful solution doesn't exceed rim of tube) and vortex for 5 seconds.
    Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the Spin Filters.

15. Load approximately 675µl onto a Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675µl of supernatant to the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x *g* for 1 minute at room temperature. **Note**: A total of three loads for each sample processed are required.

    DNA is selectively bound to the silica membrane in the Spin Filter device in the high

salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

16. Add 500µl of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x *g*.

    Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

17. Discard the flow through from the 2 ml Collection tube.
    This flow through fraction is just non-DNA organic and inorganic waste removed from the silica Spin Filter membrane by the ethanol wash solution.

18. Centrifuge at room temperature for 1 minute at 10,000 x *g*.
    This second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis.

19. Carefully place Spin Filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.

    Note: *It is important to avoid any traces of the ethanol based wash solution.*

20. Add 100µl of Solution C6 to the center of the white filter membrane.

    Note: *Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient and complete release of the DNA from the silica Spin Filter membrane. As Solution C6 (elution buffer) passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris) which lacks salt.*

    Alternatively, sterile DNA-Free PCR Grade Water may be used for DNA elution from the silica Spin Filter membrane at this step (MO BIO Catalog No. 17000-10). Solution C6 contains no EDTA. If DNA degradation is a concern, Sterile TE may also be used instead of Solution C6 for elution of DNA from the Spin Filter.

21. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

    We recommend storing DNA frozen (-20° to -80°C). Solution C6 does not contain any EDTA. To concentrate DNA see the Additional Information Section.

Thank you for choosing the PowerSoil DNA Isolation Kit.

Additional Information

Amount of Soil to Process
This kit is designed to process 0.25 g of soil. For inquiries regarding the use of larger sample amounts, please contact technical support for suggestions. For wet soils, see information under "Wet Soil Sample" below.

Wet Soil Sample
If soil sample is high in water content, remove contents from PowerBead Tube (beads and solution) and transfer into another sterile microcentrifuge tube (not provided). Add soil sample to PowerBead Tube and centrifuge at room temperature for 30 seconds at 10,000 x g. Remove as much liquid as possible with a pipet tip. Add beads and bead solution back to PowerBead Tube and follow protocol starting at step 2.

If DNA Does Not Amplify

- Make sure to check DNA yields by gel electrophoresis or spectrophotometer reading. An excess amount of DNA will inhibit a PCR reaction.
- Diluting the template DNA should not be necessary with DNA isolated with the PowerSoil DNA Isolation Kit; however, it should still be attempted.
- If DNA will still not amplify after trying the steps above, then PCR optimization (changing reaction conditions and primer choice) may be needed.

Eluted DNA Sample Is Brown

We have not observed any coloration in DNAs isolated using the PowerSoil DNA Isolation kit. If you observe coloration in your samples, please contact technical support for suggestions.

Alternative Lysis Methods

- After adding Solution C1, vortex 3-4 seconds, then heat to 70°C for 5 minutes. Vortex 3-4 seconds. Heat another 5 minutes. Vortex 3-4 seconds. This alternative procedure will reduce shearing but may also reduce yield.
- If cells are difficult to lyse, a 10 minute incubation at 70°C, after adding Solution C1, can be performed. Follow by continuing with protocol step 5.

Concentrating the DNA
The final volume of eluted DNA will be 100µl. The DNA may be concentrated by adding 4µl of 5M NaCl and inverting 3-5 times to mix. Next, add 200µl of 100% cold ethanol and invert 3-5 times to mix. Centrifuge at 10,000 x g for 5 minutes at room temperature. Decant all liquid. Remove residual ethanol in a speed vac, dessicator, or air dry. Resuspend precipitated DNA in sterile water or sterile 10 mM Tris.

DNA Floats Out of Well When Loaded on a Gel
This usually occurs because residual Solution C5 remains in the final sample. Prevent this by being careful in step 19 not to transfer liquid onto the bottom of the spin filter basket. Ethanol precipitation (described in "Concentrating the DNA") is the best way to remove residual Solution C5.

Storing DNA

DNA is eluted in Solution C6 (10mM Tris) and must be stored at -20° to -80°C to prevent degradation. DNA can be eluted in TE without loss, but the EDTA may inhibit downstream reactions such as PCR and automated sequencing. DNA may also be eluted with sterile DNA-Free PCR Grade Water (MO BIO Catalog No. 17000-10).

Other Quality Products Available from MO BIO Laboratories, Inc.

Product Description	Catalog No.
DNA Isolation Kits	
UltraClean™ Soil DNA Isolation Kit (50 preps)	12800-50
UltraClean™ Mega Soil DNA Isolation Kit (10 preps)	12900-10
UltraClean-htp™ 96 Well Soil DNA Isolation Kit (4 x 96 preps)	12896-4
UltraClean™ Fecal DNA Isolation Kit (50 preps)	12811-50
UltraClean™ Microbial DNA Isolation Kit (50 preps)	12224-50
RNA Isolation Kits	
UltraClean™ Microbial RNA Isolation Kit (50 preps)	15800-50
DNA Purification Kits	
UltraClean™ 15 DNA Purification Kit (300 preps)	12100-300
UltraClean™ GelSpin™ DNA Purification Kit (100 preps)	12400-100
UltraClean™ PCR Clean-Up™ Kit (100 preps)	12500-100

Contact Information

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Email: technical@mobio.com
Fax: 760-929-0109
Mail: Mo Bio Laboratories, Inc, 2746 Loker Ave West, Carlsbad, CA 92010

Ordering Information

Direct: Phone Mo Bio Laboratories, Inc. Toll Free 800-606-6246, or 760-929-9911
Email: orders@mobio.com
Fax: 760-929-0109
Mail: Mo Bio Laboratories, Inc, 2746 Loker Ave West, Carlsbad, CA 92010

For the distributor nearest you, visit our web site at www.mobio.com/distributors

Appendix C: Polymerase Chain Reaction Log

PCR ID	PCR Experimen Date	DNA Templat ID	Forward Primer	Forward Primer Volume	Reverse Primer	Reverse Primer Volume	Master Mix Volume	Water Vol.	Template DNA OLD	Templat DNA	Total volume	Control	Ann. Temp C	GEL Experimen Date	Band	Slide ID	Lane	Core Information
1	15-Jul-05	41	E8F	1	E533R	1	12.50	0.00	E2225 (0.1	10.5	25.00		46	15-Jul-05	X	3		Core ID# 4 Section
2	15-Jul-05	41	(f)E8F	1	(f)UA1406	1	12.50	0.00	E2225 (0.1	10.5	25.00		46	15-Jul-05	X	3		Core ID# 4 Section
3	18-Jul-05	29	E8F	1	E533R	1	12.50	0.00	1A154	10.5	25.00		46	18-Jul-05		4	2	Core ID# 4 Section
4	18-Jul-05	15	E8F	1	E533R	1	12.50	0.00	2A225	10.5	25.00		46	18-Jul-05	X	4	3	Core ID# 4 Section
5	18-Jul-05	38	E8F	1	E533R	1	12.50	0.00	C1225 (0.2	10.5	25.00		46	18-Jul-05	X	4	4	Core ID# 1 Section
6	18-Jul-05	39	E8F	1	E533R	1	12.50	0.00	C2154 (0.0	10.5	25.00		46	18-Jul-05	X	4	5	Core ID# 4 Section
7	18-Jul-05	40	E8F	1	E533R	1	12.50	0.00	E1225	10.5	25.00		46	18-Jul-05	X	4	6	Core ID# 4 Section
8	18-Jul-05	41	E8F	1	E533R	1	12.50	0.00	E2225 (0.1	10.5	25.00		46	18-Jul-05	X	4	7	Core ID# 4 Section
9	18-Jul-05	42	E8F	1	E533R	1	12.50	0.00	G1225	10.5	25.00		46	18-Jul-05		4	8	Core ID# 4 Section
10	18-Jul-05	43	E8F	1	E533R	1	12.50	0.00	G2225	10.5	25.00		46	18-Jul-05		4	9	Core ID# 4 Section
11	18-Jul-05	41	E8F	1	(f)UA1406	1	12.50	0.00	E2225 (0.1	10.5	25.00		46	18-Jul-05	X	4	10	Core ID# 4 Section
12	28-Jul-05	44	E8F	1	E533R	1	12.50	0.00	I1	10.5	25.00		46	28-Jul-05		5	1	Core ID# 4 Section
13	28-Jul-05	47	E8F	1	E533R	1	12.50	0.00	I2	10.5	25.00		46	28-Jul-05		5	2	Core ID# 4 Section
14	28-Jul-05	46	E8F	1	E533R	1	12.50	0.00	J1	10.5	25.00		46	28-Jul-05		5	3	Core ID# 4 Section
15	28-Jul-05	47	E8F	1	E533R	1	12.50	0.00	J2	10.5	25.00		46	28-Jul-05		5	4	Core ID# 4 Section
16	28-Jul-05	48	E8F	1	E533R	1	12.50	0.00	K1	10.5	25.00		46	28-Jul-05		5	5	Blank
17	28-Jul-05	49	E8F	1	E533R	1	12.50	0.00	K2	10.5	25.00		46	28-Jul-05		5	6	Blank
18	28-Jul-05	22	E8F	1	E533R	1	12.50	0.00	3A25-03	10.5	25.00		46	28-Jul-05	X	5	7	Core ID# 5 Section
19	28-Jul-05	19	E8F	1	E533R	1	12.50	0.00	3A15-03	10.5	25.00		46	28-Jul-05		5	14	Core ID# 5 Section
20	28-Jul-05	16	E8F	1	E533R	1	12.50	0.00	2A25-03	10.5	25.00		46	28-Jul-05	X	5	15	Core ID# 5 Section
21	28-Jul-05	12	E8F	1	E533R	1	12.50	0.00	2A15-03	10.5	25.00		46	28-Jul-05		5	16	Core ID# 5 Section
22	28-Jul-05	8	E8F	1	E533R	1	12.50	0.00	1B25-03	10.5	25.00		46	28-Jul-05	X	5	17	Core ID# 5 Section
23	28-Jul-05	7	E8F	1	E533R	1	12.50	0.00	1B15-03	10.5	25.00		46	28-Jul-05	X	5	18	Core ID# 5 Section
24	28-Jul-05	6	E8F	1	E533R	1	12.50	0.00	1A25-03	10.5	25.00		46	28-Jul-05	X	5	19	Core ID# 5 Section
25	28-Jul-05	3	E8F	1	E533R	1	12.50	0.00	1A15-03	10.5	25.00		46	28-Jul-05	X	5	20	Core ID# 5 Section
26	4-Aug-05	15	E8F	1	E533R	1	12.50	0.00	2A225	10.5	25.00		46	4-Aug-05		6	2	Core ID# 4 Section
27	4-Aug-05	38	E8F	1	E533R	1	12.50	0.00	C1225 (0.2	10.5	25.00		46	4-Aug-05	X	6	3	Core ID# 4 Section
28	4-Aug-05	39	E8F	1	E533R	1	12.50	0.00	C2154 (0.0	10.5	25.00		46	4-Aug-05	X	6	4	Core ID# 4 Section
29	4-Aug-05	40	E8F	1	E533R	1	12.50	0.00	E1225	10.5	25.00		46	4-Aug-05	X	6	5	Core ID# 4 Section
30	4-Aug-05	41	E8F	1	E533R	1	12.50	0.00	E2225 (0.1	10.5	25.00		46	4-Aug-05		6	6	Core ID# 4 Section
31	4-Aug-05	42	E8F	1	E533R	1	12.50	0.00	G1225	10.5	25.00		46	4-Aug-05	X	6	7	Core ID# 4 Section
32	4-Aug-05	23	E8F	1	E533R	1	12.50	0.00	3A25-03	10.5	25.00		46	4-Aug-05	X	6	8	Core ID# 5 Section
33	4-Aug-05	19	E8F	1	E533R	1	12.50	0.00	3A15-03	10.5	25.00		46	4-Aug-05	X	6	9	Core ID# 5 Section
34	4-Aug-05	16	E8F	1	E533R	1	12.50	0.00	2A25-03	10.5	25.00		46	4-Aug-05	X	6	10	Core ID# 5 Section
35	4-Aug-05	12	E8F	1	E533R	1	12.50	0.00	2A15-03	10.5	25.00		46	4-Aug-05	X	6	11	Core ID# 5 Section
36	4-Aug-05	8	E8F	1	E533R	1	12.50	0.00	1B25-03	10.5	25.00		46	4-Aug-05	X	6	12	Core ID# 5 Section
37	4-Aug-05	7	E8F	1	E533R	1	12.50	0.00	1B15-03	10.5	25.00		46	4-Aug-05	X	6	13	Core ID# 5 Section
38	4-Aug-05	6	E8F	1	E533R	1	12.50	0.00	1A25-03	10.5	25.00		46	4-Aug-05	X	6	14	Core ID# 5 Section
39	4-Aug-05	3	E8F	1	E533R	1	12.50	0.00	1A15-03	10.5	25.00		46	4-Aug-05	X	6	15	Core ID# 5 Section
40	11-Aug-05	44	E8F	1	E533R	1	12.50	5.00	I1	5.5	25.00		46	11-Aug-05	X	7	2	Core ID# 4 Section
41	11-Aug-05	46	E8F	1	E533R	1	12.50	5.00	J1	5.5	25.00		46	11-Aug-05	X	7	3	Core ID# 4 Section
42	11-Aug-05	15	E8F	1	(f)UA1406	1	12.50	0.00	2A225	10.5	25.00			11-Aug-05		7	4	Core ID# 4 Section
43	11-Aug-05	38	E8F	1	(f)UA1406	1	12.50	0.00	C1225	10.5	25.00			11-Aug-05	X	7	5	Core ID# 4 Section
44	11-Aug-05	40	E8F	1	(f)UA1406	1	12.50	0.00	E1225	10.5	25.00			11-Aug-05		7	6	Core ID# 4 Section
45	11-Aug-05	41	E8F	1	(f)UA1406	1	12.50	0.00	E2225	10.5	25.00			11-Aug-05	X	7	7	Core ID# 4 Section
46	11-Aug-05	42	E8F	1	(f)UA1406	1	12.50	0.00	G1225	10.5	25.00			11-Aug-05	X	7	8	Core ID# 4 Section
47	11-Aug-05	44	E8F	1	(f)UA1406	1	12.50	0.00	I1	10.5	25.00			11-Aug-05		7	9	Core ID# 4 Section
48	11-Aug-05	45	E8F	1	(f)UA1406	1	12.50	0.00	I2	10.5	25.00			11-Aug-05	X	7	10	Core ID# 4 Section
49	11-Aug-05	46	E8F	1	(f)UA1406	1	12.50	0.00	J1	10.5	25.00			11-Aug-05		7	11	Core ID# 4 Section
50	11-Aug-05	47	E8F	1	(f)UA1406	1	12.50	0.00	J2	10.5	25.00			11-Aug-05	X	7	12	Core ID# 4 Section
51	11-Aug-05	48	E8F	1	(f)UA1406	1	12.50	0.00	K1	10.5	25.00			11-Aug-05		7	13	Blank
52	12-Aug-05	15	E8F	1	(f)UA1406	1	12.50	0.00	2A225	10.5	25.00		46	12-Aug-05		8		Core ID# 4 Section
53	12-Aug-05	38	E8F	1	(f)UA1406	1	12.50	0.00	C1225	10.5	25.00		46	12-Aug-05	X	8		Core ID# 4 Section
54	12-Aug-05	40	E8F	1	(f)UA1406	1	12.50	0.00	E1225	10.5	25.00		46	12-Aug-05		8		Core ID# 4 Section
55	12-Aug-05	41	E8F	1	(f)UA1406	1	12.50	0.00	E2225	10.5	25.00		46	12-Aug-05	X	8		Core ID# 4 Section
56	12-Aug-05	42	E8F	1	(f)UA1406	1	12.50	0.00	G1225	10.5	25.00		46	12-Aug-05	X	8		Core ID# 4 Section
57	12-Aug-05	44	E8F	1	(f)UA1406	1	12.50	0.00	I1	10.5	25.00		46	12-Aug-05		8		Core ID# 4 Section
58	12-Aug-05	45	E8F	1	(f)UA1406	1	12.50	0.00	I2	10.5	25.00		46	12-Aug-05		8		Core ID# 4 Section
59	12-Aug-05	46	E8F	1	(f)UA1406	1	12.50	0.00	J1	10.5	25.00		46	12-Aug-05		8		Core ID# 4 Section
60	12-Aug-05	47	E8F	1	(f)UA1406	1	12.50	0.00	J2	10.5	25.00		46	12-Aug-05		8		Core ID# 4 Section
61	12-Aug-05	48	E8F	1	(f)UA1406	1	12.50	0.00	K1	10.5	25.00	X	46	12-Aug-05		8		Blank
62	12-Aug-05	3	E8F	1	(f)UA1406	1	12.50	0.00	1A1503	10.5	25.00		46	12-Aug-05	X	8		Core ID# 5 Section
63	12-Aug-05	6	E8F	1	(f)UA1406	1	12.50	0.00	1A2503	10.5	25.00		46	12-Aug-05	X	8		Core ID# 5 Section
64	12-Aug-05	7	E8F	1	(f)UA1406	1	12.50	0.00	1B1503	10.5	25.00		46	12-Aug-05	X	8		Core ID# 5 Section
65	12-Aug-05	8	E8F	1	(f)UA1406	1	12.50	0.00	1B2503	10.5	25.00		46	12-Aug-05		8		Core ID# 5 Section
66	12-Aug-05	12	E8F	1	(f)UA1406	1	12.50	0.00	2A1503	10.5	25.00		46	12-Aug-05	X	8		Core ID# 5 Section
67	12-Aug-05	16	E8F	1	(f)UA1406	1	12.50	0.00	2A2503	10.5	25.00		46	12-Aug-05	X	8		Core ID# 5 Section
68	12-Aug-05	11	E8F	1	(f)UA1406	1	12.50	0.00	3A1503	10.5	25.00		46	12-Aug-05		8		Core ID# 5 Section
69	12-Aug-05	22	E8F	1	(f)UA1406	1	12.50	0.00	3A2503	10.5	25.00		46	12-Aug-05	X	8		Core ID# 5 Section
70	12-Sep-05	2	(f)E8F	1	(f)UA1406	1	12.50	0.00	1A1	10.5	25.00		46	12-Sep-05		9		Core ID# 6 Section
71	12-Sep-05	5	(f)E8F	1	(f)UA1406	1	12.50	0.00	1A2	10.5	25.00		46	12-Sep-05		9		Core ID# 6 Section
72	12-Sep-05	10	(f)E8F	1	(f)UA1406	1	12.50	0.00	2A1	10.5	25.00		46	12-Sep-05		9		Core ID# 6 Section

PCR ID	PCR Experimer Date	DNA Templa ID	Forward Primer	Forwarc Primer Volume	Reverse Primer	Reverse Primer Volume	Master Mix Volume	Water Vol.	Template DNA OLD	Templa DNA	Total volume	Control	Ann. Temp C	GEL Experimer Date	Banc	Slide ID	Lane	Core Information
73	12-Sep-05	14	(f)E8F	1	(f)UA1406	1	12.50	0.00	2A2	10.5	25.00		46	12-Sep-05		9		Core ID# 6 Section
74	12-Sep-05	18	(f)E8F	1	(f)UA1406	1	12.50	0.00	3A1	10.5	25.00		46	12-Sep-05	X	9		Core ID# 6 Section
75	12-Sep-05	21	(f)E8F	1	(f)UA1406	1	12.50	0.00	3A2	10.5	25.00		46	12-Sep-05	X	9		Core ID# 6 Section
76	12-Sep-05	1	(f)E8F	1	(f)UA1406	1	12.50	0.00	1A1	10.5	25.00		46	12-Sep-05	X	9		Core ID# 7 Section
77	12-Sep-05	4	(f)E8F	1	(f)UA1406	1	12.50	0.00	1A2	10.5	25.00		46	12-Sep-05	X	9		Core ID# 7 Section
78	12-Sep-05	9	(f)E8F	1	(f)UA1406	1	12.50	0.00	2A1	10.5	25.00		46	12-Sep-05	X	9		Core ID# 7 Section
79	12-Sep-05	13	(f)E8F	1	(f)UA1406	1	12.50	0.00	2A2	10.5	25.00		46	12-Sep-05	X	9		Core ID# 7 Section
80	12-Sep-05	17	(f)E8F	1	(f)UA1406	1	12.50	0.00	3A1	10.5	25.00		46	12-Sep-05	X	9		Core ID# 7 Section
81	12-Sep-05	20	(f)E8F	1	(f)UA1406	1	12.50	0.00	3A2	10.5	25.00		46	12-Sep-05	X	9		Core ID# 7 Section
82	12-Sep-05		(f)E8F	1	(f)UA1406	1	12.50	9.50		10.5	34.50	X	46	12-Sep-05		9		Blank
83	13-Sep-05	2	(f)E8F	1	(f)UA1406	1	12.50	0.00	1A1	10.5	25.00		46	13-Sep-05		10		Core ID# 6 Section
84	13-Sep-05	5	(f)E8F	1	(f)UA1406	1	12.50	0.00	1A2	10.5	25.00		46	13-Sep-05		10		Core ID# 6 Section
85	13-Sep-05	10	(f)E8F	1	(f)UA1406	1	12.50	0.00	2A1	10.5	25.00		46	13-Sep-05		10		Core ID# 6 Section
86	13-Sep-05	14	(f)E8F	1	(f)UA1406	1	12.50	0.00	2A2	10.5	25.00		46	13-Sep-05		10		Core ID# 6 Section
87	13-Sep-05	18	(f)E8F	1	(f)UA1406	1	12.50	0.00	3A1	10.5	25.00		46	13-Sep-05	X	10		Core ID# 6 Section
88	13-Sep-05	21	(f)E8F	1	(f)UA1406	1	12.50	0.00	3A2	10.5	25.00		46	13-Sep-05	X	10		Core ID# 6 Section
89	13-Sep-05	1	(f)E8F	1	(f)UA1406	1	12.50	0.00	1A1	10.5	25.00		46	13-Sep-05	X	10		Core ID# 7 Section
90	13-Sep-05	4	(f)E8F	1	(f)UA1406	1	12.50	0.00	1A2	10.5	25.00		46	13-Sep-05	X	10		Core ID# 7 Section
91	13-Sep-05	9	(f)E8F	1	(f)UA1406	1	12.50	0.00	2A1	10.5	25.00		46	13-Sep-05	X	10		Core ID# 7 Section
92	13-Sep-05	13	(f)E8F	1	(f)UA1406	1	12.50	0.00	2A2	10.5	25.00		46	13-Sep-05	X	10		Core ID# 7 Section
93	13-Sep-05	17	(f)E8F	1	(f)UA1406	1	12.50	0.00	3A1	10.5	25.00		46	13-Sep-05	X	10		Core ID# 7 Section
94	13-Sep-05	20	(f)E8F	1	(f)UA1406	1	12.50	0.00	3A2	10.5	25.00		46	13-Sep-05	X	10		Core ID# 7 Section
95	13-Sep-05		(f)E8F	1	(f)UA1406	1	12.50	10.50			25.00	X	46	13-Sep-05		10		Blank
96	20-Sep-05	15	(f)E8F	1	(f)UA1406	1	12.50	5.00	2A225	5.5	25.00		46	20/13/2005		11		Core ID# 4 Section
97	20-Sep-05	40	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.00	E1225	5.50	25.00		46	20-Sep-05		11		Core ID# 4 Section
98	20-Sep-05	33	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.00	A5	5.50	25.00		46	20-Sep-05		11		Core ID# 4 Section
99	20-Sep-05	34	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.00	A6	5.50	25.00		46	20-Sep-05		11		Core ID# 4 Section
100	20-Sep-05	3	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	1A1503	10.50	25.00		46	20-Sep-05		11		Core ID# 5 Section
101	20-Sep-05	6	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	1A2503	10.50	25.00		46	20-Sep-05		11		Core ID# 5 Section
102	20-Sep-05	7	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	1B1503	10.50	25.00		46	20-Sep-05		11		Core ID# 5 Section
103	20-Sep-05	8	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.00	1B2503	5.50	25.00		46	20-Sep-05		11		Core ID# 5 Section
104	20-Sep-05	12	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	2A1503	10.50	25.00		46	20-Sep-05		11		Core ID# 5 Section
105	20-Sep-05	16	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	2A2503	10.50	25.00		46	20-Sep-05		11		Core ID# 5 Section
106	20-Sep-05	19	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	3A1503	10.50	25.00		46	20-Sep-05		11		Core ID# 5 Section
107	20-Sep-05	22	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	3A2503	10.50	25.00		46	20-Sep-05		11		Core ID# 5 Section
108	20-Sep-05	26	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A13	10.50	25.00		46	20-Sep-05		11		Core ID# 1 Section
109	20-Sep-05	27	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A14	10.50	25.00		46	20-Sep-05		11		Core ID# 1 Section
110	20-Sep-05	28	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A15	10.50	25.00		46	20-Sep-05		11		Core ID# 1 Section
111	20-Sep-05	30	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A16	10.50	25.00		46	20-Sep-05		11		Core ID# 1 Section
112	20-Sep-05	31	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A17	10.50	25.00		46	20-Sep-05		11		Core ID# 1 Section
113	20-Sep-05	32	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A18	10.50	25.00		46	20-Sep-05		11		Core ID# 1 Section
114	20-Sep-05	35	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A7	10.50	25.00		46	20-Sep-05		11		Core ID# 2 Section
115	20-Sep-05	36	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A8	10.50	25.00		46	20-Sep-05		11		Core ID# 2 Section
116	20-Sep-05	37	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A9	10.50	25.00		46	20-Sep-05		11		Core ID# 2 Section
117	20-Sep-05	23	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A10	10.50	25.00		46	20-Sep-05		11		Core ID# 2 Section
118	20-Sep-05	24	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A11	10.50	25.00		46	20-Sep-05		11		Core ID# 2 Section
119	20-Sep-05	25	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A12	10.50	25.00		46	20-Sep-05		11		Core ID# 2 Section
120	20-Sep-05	5	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.00	1A2	5.50	25.00		46	20-Sep-05		11		Core ID# 6 Section
121	20-Sep-05	9	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.00	2A1	5.50	25.00		46	20-Sep-05		11		Core ID# 7 Section
122	20-Sep-05	13	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.00	2A2	5.50	25.00		46	20-Sep-05		11		Core ID# 7 Section
123	20-Sep-05	17	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.00	3A1	5.50	25.00		46	20-Sep-05		11		Core ID# 7 Section
124	20-Sep-05		(f)E8F	1.00	(f)UA1406	1.00	12.50	10.50			25.00	X	46	20-Sep-05		11		Blank
125	22-Sep-05	35	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A7	10.50	25.00		46	22-Sep-05	X	12		Core ID# 2 Section
126	22-Sep-05	36	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A8	10.50	25.00		46	22-Sep-05	X	12		Core ID# 2 Section
127	22-Sep-05	37	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A9	10.50	25.00		46	22-Sep-05	X	12		Core ID# 2 Section
128	22-Sep-05	23	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A10	10.50	25.00		46	22-Sep-05	X	12		Core ID# 2 Section
129	22-Sep-05	24	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A11	10.50	25.00		46	22-Sep-05	X	12		Core ID# 2 Section
130	22-Sep-05	25	(f)E8F	1.00	(f)UA1406	1.00	12.50	0.00	A12	10.50	25.00		46	22-Sep-05	X	12		Core ID# 2 Section
131	22-Sep-05	3	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.50	1A1503	5.00	25.00		46	22-Sep-05	X	12		Core ID# 5 Section
132	22-Sep-05	6	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.50	1A2503	5.00	25.00		46	22-Sep-05	X	12		Core ID# 5 Section
133	22-Sep-05	7	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.50	1B1503	5.00	25.00		46	22-Sep-05	X	12		Core ID# 5 Section
134	22-Sep-05	8	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.50	1B2503	5.00	25.00		46	22-Sep-05	X	12		Core ID# 5 Section
135	22-Sep-05	12	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.50	2A1503	5.00	25.00		46	22-Sep-05	X	12		Core ID# 5 Section
136	22-Sep-05	16	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.50	2A2503	5.00	25.00		46	22-Sep-05	X	12		Core ID# 5 Section
137	22-Sep-05	19	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.50	3A1503	5.00	25.00		46	22-Sep-05	X	12		Core ID# 5 Section
138	22-Sep-05	22	(f)E8F	1.00	(f)UA1406	1.00	12.50	5.50	3A2503	5.00	25.00		46	22-Sep-05	X	12		Core ID# 5 Section
139			(f)E8F	1.00	(f)UA1406	1.00	12.50	10.50			25.00	X	46			12		Blank
140	11-Oct-05	78	E8F	1.00	E533R	1.00	12.50	0.00	1A064	10.5	25.00		46	12-Oct-05		13	2	Core ID# 7 Section
141	11-Oct-05	78	E8F	1.00	E533R	1.00	12.50	1.50	1A064	9	25.00		46	12-Oct-05		13	3	Core ID# 7 Section

PCR ID	PCR Experimer Date	DNA Templa ID	Forward Primer	Forward Primer Volume	Reverse Primer	Reverse Primer Volume	Master Mix Volume	Water Vol.	Template DNA OLD	Templa DNA	Total volume	Control	Ann. Temp C	GEL Experimer Date	Banc	Slide ID	Lane	Core Information
142	11-Oct-05	78	E8F	1.00	E533R	1.00	12.50	3.50	1A064	7	25.00		46	12-Oct-05	X	13	4	Core ID# 7 Section
143	11-Oct-05	78	E8F	1.00	E533R	1.00	12.50	5.50	1A064	5	25.00		46	12-Oct-05	X	13	5	Core ID# 7 Section
144	11-Oct-05	78	E8F	1.00	E533R	1.00	12.50	7.50	1A064	3	25.00		46	12-Oct-05	X	13	6	Core ID# 7 Section
145	12-Oct-05	50	E8F	1.00	E533R	1.00	12.50	0.00	001	10.5	25.00		46	13-Oct-05		14	2	Core ID# 2 Section
146	12-Oct-05	50	E8F	1.00	E533R	1.00	12.50	1.50	001	9	25.00		46	13-Oct-05		14	3	Core ID# 2 Section
147	12-Oct-05	50	E8F	1.00	E533R	1.00	12.50	3.50	001	7	25.00		46	13-Oct-05	X	14	4	Core ID# 2 Section
148	12-Oct-05	50	E8F	1.00	E533R	1.00	12.50	5.50	001	5	25.00		46	13-Oct-05	X	14	5	Core ID# 2 Section
149	12-Oct-05	50	E8F	1.00	E533R	1.00	12.50	7.50	001	3	25.00		46	13-Oct-05	X	14	6	Core ID# 2 Section
150	12-Oct-05	51	E8F	1.00	E533R	1.00	12.50	0.00	002	10.5	25.00		46	13-Oct-05	X	14	7	Core ID# 2 Section
151	12-Oct-05	51	E8F	1.00	E533R	1.00	12.50	1.50	002	9	25.00		46	13-Oct-05		14	8	Core ID# 2 Section
152	12-Oct-05	51	E8F	1.00	E533R	1.00	12.50	3.50	002	7	25.00		46	13-Oct-05	X	14	9	Core ID# 2 Section
153	12-Oct-05	51	E8F	1.00	E533R	1.00	12.50	5.50	002	5	25.00		46	13-Oct-05		14	10	Core ID# 2 Section
154	12-Oct-05	51	E8F	1.00	E533R	1.00	12.50	7.50	002	3	25.00		46	13-Oct-05	X	14	11	Core ID# 2 Section
155	12-Oct-05	52	E8F	1.00	E533R	1.00	12.50	0.00	003	10.5	25.00		46	13-Oct-05	X	14	12	Core ID# 2 Section
156	12-Oct-05	52	E8F	1.00	E533R	1.00	12.50	1.50	003	9	25.00		46	13-Oct-05		14	13	Core ID# 2 Section
157	12-Oct-05	52	E8F	1.00	E533R	1.00	12.50	3.50	003	7	25.00		46	13-Oct-05		14	14	Core ID# 2 Section
158	12-Oct-05	52	E8F	1.00	E533R	1.00	12.50	5.50	003	5	25.00		46	13-Oct-05	X	14	15	Core ID# 2 Section
159	12-Oct-05	52	E8F	1.00	E533R	1.00	12.50	7.50	003	3	25.00		46	13-Oct-05	X	14	16	Core ID# 2 Section
160	12-Oct-05	53	E8F	1.00	E533R	1.00	12.50	0.00	004	10.5	25.00		46	13-Oct-05		14	17	Core ID# 2 Section
161	12-Oct-05	53	E8F	1.00	E533R	1.00	12.50	1.50	004	9	25.00		46	13-Oct-05		14	18	Core ID# 2 Section
162	12-Oct-05	53	E8F	1.00	E533R	1.00	12.50	3.50	004	7	25.00		46	13-Oct-05		14	19	Core ID# 2 Section
163	12-Oct-05	53	E8F	1.00	E533R	1.00	12.50	5.50	004	5	25.00		46	13-Oct-05	X	14	20	Core ID# 2 Section
164	12-Oct-05	53	E8F	1.00	E533R	1.00	12.50	7.50	004	3	25.00		46	13-Oct-05	X	14	2	Core ID# 2 Section
165	12-Oct-05	54	E8F	1.00	E533R	1.00	12.50	0.00	005	10.5	25.00		46	13-Oct-05		14	3	Core ID# 2 Section
166	12-Oct-05	54	E8F	1.00	E533R	1.00	12.50	1.50	005	9	25.00		46	13-Oct-05	X	14	4	Core ID# 2 Section
167	12-Oct-05	54	E8F	1.00	E533R	1.00	12.50	3.50	005	7	25.00		46	13-Oct-05	X	14	5	Core ID# 2 Section
168	12-Oct-05	54	E8F	1.00	E533R	1.00	12.50	5.50	005	5	25.00		46	13-Oct-05	X	14	6	Core ID# 2 Section
169	12-Oct-05	54	E8F	1.00	E533R	1.00	12.50	7.50	005	3	25.00		46	13-Oct-05	X	14	7	Core ID# 2 Section
170	12-Oct-05	55	E8F	1.00	E533R	1.00	12.50	0.00	006	10.5	25.00		46	13-Oct-05	X	14	8	Core ID# 2 Section
171	12-Oct-05	55	E8F	1.00	E533R	1.00	12.50	1.50	006	9	25.00		46	13-Oct-05	X	14	9	Core ID# 2 Section
172	12-Oct-05	55	E8F	1.00	E533R	1.00	12.50	3.50	006	7	25.00		46	13-Oct-05	X	14	10	Core ID# 2 Section
173	12-Oct-05	55	E8F	1.00	E533R	1.00	12.50	5.50	006	5	25.00		46	13-Oct-05	X	14	11	Core ID# 2 Section
174	12-Oct-05	55	E8F	1.00	E533R	1.00	12.50	7.50	006	3	25.00		46	13-Oct-05	X	14	12	Core ID# 2 Section
175	12-Oct-05	56	E8F	1.00	E533R	1.00	12.50	0.00	007	10.5	25.00	X	46	13-Oct-05	X	14	13	Core ID# 2
176	12-Oct-05	56	E8F	1.00	E533R	1.00	12.50	1.50	007	9	25.00	X	46	13-Oct-05	X	14	14	Core ID# 2
177	12-Oct-05	56	E8F	1.00	E533R	1.00	12.50	3.50	007	7	25.00	X	46	13-Oct-05		14	15	Core ID# 2
178	12-Oct-05	56	E8F	1.00	E533R	1.00	12.50	5.50	007	5	25.00	X	46	13-Oct-05	X	14	16	Core ID# 2
179	12-Oct-05	56	E8F	1.00	E533R	1.00	12.50	7.50	007	3	25.00	X	46	13-Oct-05	X	14	17	Core ID# 2
180	12-Oct-05	57	E8F	1.00	E533R	1.00	12.50	0.00	008	10.5	25.00	X	46	13-Oct-05		14	18	Core ID# 2
181	12-Oct-05	57	E8F	1.00	E533R	1.00	12.50	1.50	008	9	25.00	X	46	13-Oct-05		14	19	Core ID# 2
182	12-Oct-05	57	E8F	1.00	E533R	1.00	12.50	3.50	008	7	25.00	X	46	13-Oct-05		14	20	Core ID# 2
183	12-Oct-05	57	E8F	1.00	E533R	1.00	12.50	5.50	008	5	25.00	X	46		n/a	n/a		Core ID# 2
184	12-Oct-05	57	E8F	1.00	E533R	1.00	12.50	7.50	008	3	25.00	X	46		n/a	n/a		Core ID# 2
185	18-Oct-05	35	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05	X	15	2	Core ID# 2 Section
186	18-Oct-05	36	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05		15	3	Core ID# 2 Section
187	18-Oct-05	37	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05	X	15	4	Core ID# 2 Section
188	18-Oct-05	23	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05	X	15	5	Core ID# 2 Section
189	18-Oct-05	24	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05		15	6	Core ID# 2 Section
190	18-Oct-05	25	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05		15	7	Core ID# 2 Section
191	18-Oct-05	6	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05		15	8	Core ID# 5 Section
192	18-Oct-05	7	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05	X	15	9	Core ID# 5 Section
193	18-Oct-05	8	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05	X	15	10	Core ID# 5 Section
194	18-Oct-05	11	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05		15	11	Core ID# 5 Section
195	18-Oct-05	12	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05	X	15	12	Core ID# 5 Section
196	18-Oct-05	19	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05	X	15	13	Core ID# 5 Section
197	18-Oct-05	22	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	18-Oct-05		15	14	Core ID# 5 Section
198	24-Oct-05	2	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 6 Section
199	24-Oct-05	5	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 6 Section
200	24-Oct-05	10	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 6 Section
201	24-Oct-05	14	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 6 Section
202	24-Oct-05	18	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 6 Section
203	24-Oct-05	21	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 6 Section
204	24-Oct-05	26	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 1 Section
205	24-Oct-05	27	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 1 Section
206	24-Oct-05	28	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 1 Section
207	24-Oct-05	30	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 1 Section
208	24-Oct-05	31	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 1 Section
209	24-Oct-05	32	(f)E8F	1.00	(f)UA1406	1.00	12.50	3.50		7	25.00		46	24-Oct-05		16		Core ID# 1 Section
210	24-Oct-05	H2O	(f)E8F	2.00	(f)UA1406	1.00	12.50	9.50		0	25.00			24-Oct-05		16		Blank

PCR ID	PCR Experimer Date	DNA Templa ID	Forward Primer	Forward Primer Volume	Reverse Primer	Reverse Primer Volume	Master Mix Volume	Water Vol.	Template DNA OLD	Templa DNA	Total volume	Control	Ann. Temp C	GEL Experimer Date	Banc	Slide ID	Lane	Core Information
211	25-Oct-05	2	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05	X	17	2	Core ID# 6 Section
212	25-Oct-05	5	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05	X	17	3	Core ID# 6 Section
213	25-Oct-05	10	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05		17	4	Core ID# 6 Section
214	25-Oct-05	14	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05		17	5	Core ID# 6 Section
215	25-Oct-05	18	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05		17	6	Core ID# 6 Section
216	25-Oct-05	21	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05		17	7	Core ID# 6 Section
217	25-Oct-05	26	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05	X	17	8	Core ID# 1 Section
218	25-Oct-05	27	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05		17	9	Core ID# 1 Section
219	25-Oct-05	28	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05		17	10	Core ID# 1 Section
220	25-Oct-05	30	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05		17	11	Core ID# 1 Section
221	25-Oct-05	31	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05	X	17	12	Core ID# 1 Section
222	25-Oct-05	32	(f)E8F	1.00	(f)UA1406	1.00	12.50	7.50		3	25.00		46	25-Oct-05	X	17	13	Core ID# 1 Section
223	25-Oct-05	H2O	(f)E8F	2.00	(f)UA1406	1.00	12.50	9.50		0	25.00			25-Oct-05		17	14	Blank
224	25-Oct-05	61	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	25-Oct-05	X	18	2	Core ID# 1 Section
225	25-Oct-05	62	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	25-Oct-05		18	3	Core ID# 1 Section
226	25-Oct-05	63	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	25-Oct-05	X	18	4	Core ID# 1 Section
227	25-Oct-05	64	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	25-Oct-05		18	5	Core ID# 5 Section
228	25-Oct-05	65	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	25-Oct-05	X	18	6	Core ID# 5 Section
229	25-Oct-05	66	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	25-Oct-05		18	7	Core ID# 5 Section
230	25-Oct-05	73	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00	X	46	25-Oct-05		18	8	Core ID# 5
231	25-Oct-05	H2O	E8F	1.00	E533R	1.00	12.50	10.50		0	25.00	X	46	25-Oct-05		18	9	Blank
232	26-Oct-05	61	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05	X	19	2	Core ID# 1 Section
233	26-Oct-05	62	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05		19	3	Core ID# 1 Section
234	26-Oct-05	63	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05		19	4	Core ID# 1 Section
235	26-Oct-05	64	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05	X	19	5	Core ID# 5 Section
236	26-Oct-05	65	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05		19	6	Core ID# 5 Section
237	26-Oct-05	66	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05		19	7	Core ID# 5 Section
238	26-Oct-05	67	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05	X	19	8	Core ID# 6 Section
239	26-Oct-05	68	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05	X	19	9	Core ID# 6 Section
240	26-Oct-05	69	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Oct-05	X	19	10	Core ID# 6 Section
241	26-Oct-05	H2O	E8F	1.00	E533R	1.00	12.50	10.50		0	25.00	X	46	27-Oct-05		19	11	Blank
242	26-Oct-05	73	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00	X	46	27-Oct-05		19	12	Core ID# 5
243	31-Oct-05	28	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	1-Nov-05	X	20	2	Core ID# 1 Section
244	31-Oct-05	50	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	1-Nov-05	X	20	3	Core ID# 2 Section
245	31-Oct-05	52	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	1-Nov-05		20	4	Core ID# 2 Section
246	31-Oct-05	54	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	1-Nov-05	X	20	5	Core ID# 2 Section
247	31-Oct-05	56	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	1-Nov-05		20	6	Core ID# 2
248	31-Oct-05	63	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	1-Nov-05		20	7	Core ID# 1 Section
249	31-Oct-05	H2O	E8F	1.00	E533R	1.00	12.50	10.50		0	25.00	X	46	1-Nov-05		20	8	Blank
250	11-Nov-05	33	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	2	Core ID# 4 Section
251	11-Nov-05	33	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	13-Nov-05	X	21	3	Core ID# 4 Section
252	11-Nov-05	33	E8F	1.00	E533R	1.00	12.50	3.00		7.5	25.00		46	13-Nov-05	X	21	4	Core ID# 4 Section
253	11-Nov-05	38	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	5	Core ID# 4 Section
254	11-Nov-05	38	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	6	Core ID# 4 Section
255	11-Nov-05	40	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	7	Core ID# 4 Section
256	11-Nov-05	40	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	8	Core ID# 4 Section
257	11-Nov-05	53	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	9	Core ID# 2 Section
258	11-Nov-05	54	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	10	Core ID# 2 Section
259	11-Nov-05	58	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	11	Core ID# 2 Section
260	11-Nov-05	59	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	12	Core ID# 2 Section
261	11-Nov-05	60	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	13	Core ID# 2 Section
262	11-Nov-05	61	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	14	Core ID# 1 Section
263	11-Nov-05	62	E8F	1.00	E533R	1.00	12.50	3.00		7.5	25.00		46	13-Nov-05		21	15	Core ID# 1 Section
264	11-Nov-05	62	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	13-Nov-05	X	21	16	Core ID# 1 Section
265	11-Nov-05	63	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	17	Core ID# 1 Section
266	11-Nov-05	64	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	18	Core ID# 5 Section
267	11-Nov-05	65	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	19	Core ID# 5 Section
268	11-Nov-05	66	E8F	1.00	E533R	1.00	12.50	3.00		7.5	25.00		46	13-Nov-05		21	20	Core ID# 5 Section
269	11-Nov-05	66	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	13-Nov-05		21	1B	Core ID# 5 Section
270	11-Nov-05	67	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	2B	Core ID# 6 Section
271	11-Nov-05	68	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	3B	Core ID# 6 Section
272	11-Nov-05	69	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	12B	Core ID# 6 Section
273	11-Nov-05	70	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	13B	Core ID# 7 Section
274	11-Nov-05	71	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	14B	Core ID# 7 Section
275	11-Nov-05	72	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	13-Nov-05	X	21	15B	Core ID# 7 Section
276	11-Nov-05	H2O	E8F	1.00	E533R	1.00	12.50	10.50		0	25.00	X	46	13-Nov-05		21	16B	Blank
277	11-Nov-05	H2O	E8F	1.00	E533R	1.00	12.50	10.50		0	25.00	X	46	13-Nov-05		21	17B	Blank
278	13-Nov-05	62	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	14-Nov-05	X	22	2	Core ID# 1 Section
279	13-Nov-05	58	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	14-Nov-05	X	22	3	Core ID# 2 Section

PCR ID	PCR Experimer Date	DNA Templa ID	Forward Primer	Forward Primer Volume	Reverse Primer	Reverse Primer Volume	Master Mix Volume	Water Vol.	Template DNA OLD	Templa DNA	Total volume	Control	Ann. Temp C	GEL Experimer Date	Banc	Slide ID	Lane	Core Information
280	13-Nov-05	59	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	14-Nov-05	X	22	4	Core ID# 2 Section
281	13-Nov-05	70	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	14-Nov-05		22	5	Core ID# 7 Section
281	13-Nov-05	70	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Dec-05		31	18	Core ID# 7 Section
282	13-Nov-05	71	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	14-Nov-05		22	6	Core ID# 7 Section
282	13-Nov-05	71	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	27-Dec-05		31	19	Core ID# 7 Section
283	13-Nov-05	72	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	14-Nov-05	X	22	7	Core ID# 7 Section
284	13-Nov-05	19	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	14-Nov-05	X	22	8	Core ID# 5 Section
285	13-Nov-05	19	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	14-Nov-05	X	22	9	Core ID# 5 Section
286	13-Nov-05	22	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	14-Nov-05	X	22	10	Core ID# 5 Section
287	13-Nov-05	22	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	14-Nov-05	X	22	1b	Core ID# 5 Section
288	13-Nov-05	H2O	E8F	1.00	E533R	1.00	12.50	10.50		0	25.00	X	46	14-Nov-05		22	2b	Blank
289	13-Nov-05	H2O	E8F	1.00	E533R	1.00	12.50	10.50		0	25.00	X	46	14-Nov-05		22	3b	Blank
290	27-Dec-05	68	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	27-Dec-05		31	2	Core ID# 6 Section
291	27-Dec-05	102	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	27-Dec-05		31	3	Core ID# 8 Section
292	27-Dec-05	103	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	27-Dec-05		31	4	Core ID# 8 Section
293	27-Dec-05	104	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	27-Dec-05	X	31	5	Core ID# 8 Section
294	27-Dec-05	105	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	27-Dec-05	X	31	6	Core ID# 9 Section
295	27-Dec-05	106	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	27-Dec-05	X	31	7	Core ID# 9 Section
296	27-Dec-05	107	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	27-Dec-05	X	31	8	Core ID# 9 Section
297	27-Dec-05	108	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00	X	46	27-Dec-05	X	31	9	Blank
298	27-Dec-05	68	E8F	1.00	E533R	1.00	12.50	5.00		5.5	25.00		46	27-Dec-05	X	31	10	Core ID# 6 Section
299	27-Dec-05	102	E8F	1.00	E533R	1.00	12.50	5.00		5.5	25.00		46	27-Dec-05	X	31	11	Core ID# 8 Section
300	27-Dec-05	103	E8F	1.00	E533R	1.00	12.50	5.00		5.5	25.00		46	27-Dec-05		31	12	Core ID# 8 Section
301	27-Dec-05	104	E8F	1.00	E533R	1.00	12.50	5.00		5.5	25.00		46	27-Dec-05	X	31	13	Core ID# 8 Section
302	27-Dec-05	105	E8F	1.00	E533R	1.00	12.50	5.00		5.5	25.00		46	27-Dec-05		31	14	Core ID# 9 Section
303	27-Dec-05	106	E8F	1.00	E533R	1.00	12.50	5.00		5.5	25.00		46	27-Dec-05	X	31	15	Core ID# 9 Section
304	27-Dec-05	107	E8F	1.00	E533R	1.00	12.50	5.00		5.5	25.00		46	27-Dec-05	X	31	16	Core ID# 9 Section
305	27-Dec-05	108	E8F	1.00	E533R	1.00	12.50	5.00		5.5	25.00	X	46	27-Dec-05	X	31	17	Blank
306	29-Dec-05	109	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	30-Dec-05	X	32	2	Core ID# 8 Section
307	29-Dec-05	109	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	30-Dec-05	X	32	3	Core ID# 8 Section
308	29-Dec-05	110	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	30-Dec-05	X	32	4	Core ID# 8 Section
309	29-Dec-05	110	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	30-Dec-05	X	32	5	Core ID# 8 Section
310	29-Dec-05	111	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	30-Dec-05	X	32	6	Core ID# 8 Section
311	29-Dec-05	111	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	30-Dec-05	X	32	7	Core ID# 8 Section
312	29-Dec-05	112	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	30-Dec-05	X	32	8	Core ID# 9 Section
313	29-Dec-05	112	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	30-Dec-05	X	32	9	Core ID# 9 Section
314	29-Dec-05	113	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	30-Dec-05	X	32	10	Core ID# 9 Section
315	29-Dec-05	113	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	30-Dec-05	X	32	11	Core ID# 9 Section
316	29-Dec-05	114	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	30-Dec-05	X	32	12	Core ID# 9 Section
317	29-Dec-05	114	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	30-Dec-05	X	32	13	Core ID# 9 Section
318	29-Dec-05	115	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	30-Dec-05	X	32	14	Core ID# 7 Section
319	29-Dec-05	115	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	30-Dec-05	X	32	15	Core ID# 7 Section
320	29-Dec-05	116	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	30-Dec-05	X	32	16	Core ID# 7 Section
321	29-Dec-05	116	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	30-Dec-05	X	32	17	Core ID# 7 Section
322	29-Dec-05	117	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00	X	46	30-Dec-05	X	32	18	Blank
323	29-Dec-05	117	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00	X	46	30-Dec-05	X	32	19	Blank
324	30-Dec-05	118	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	31-Dec-05	X	35	2	Core ID# 8 Section
325	30-Dec-05	118	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	3	Core ID# 8 Section
326	30-Dec-05	119	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	31-Dec-05	X	35	4	Core ID# 8 Section
327	30-Dec-05	119	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	5	Core ID# 8 Section
328	30-Dec-05	120	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	31-Dec-05	X	35	6	Core ID# 8 Section
329	30-Dec-05	120	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	7	Core ID# 8 Section
330	30-Dec-05	121	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	31-Dec-05	X	35	8	Core ID# 9 Section
331	30-Dec-05	121	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	9	Core ID# 9 Section
332	30-Dec-05	122	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	31-Dec-05	X	35	10	Core ID# 9 Section
333	30-Dec-05	122	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	11	Core ID# 9 Section
334	30-Dec-05	123	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	31-Dec-05	X	35	12	Core ID# 9 Section
335	30-Dec-05	123	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	13	Core ID# 9 Section
336	30-Dec-05	124	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	31-Dec-05	X	35	14	Core ID# 7 Section
337	30-Dec-05	124	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	15	Core ID# 7 Section
338	30-Dec-05	125	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	31-Dec-05	X	35	16	Core ID# 7 Section
339	30-Dec-05	125	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	17	Core ID# 7 Section
340	30-Dec-05	126	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00	X	46	31-Dec-05	X	35	18	Blank
341	30-Dec-05	126	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00	X	46	31-Dec-05	X	35	19	Blank
342	30-Dec-05	68	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05		35	20	Core ID# 6 Section
343	30-Dec-05	68	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	31-Dec-05	X	35	22	Core ID# 6 Section
344	2-Jan-06	127	E8F	1.00	E533R	1.00	12.50	8.50		2	25.00	X	46	2-Jan-06		36	2	Blank
345	2-Jan-06	127	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00	X	46	2-Jan-06		36	3	Blank
346	2-Jan-06	127	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00	X	46	2-Jan-06		36	4	Blank

PCR ID	PCR Experime Date	DNA Templa ID	Forwar Primer	Forwar Primer Volume	Reverse Primer	Revers Primer Volum	Master Mix Volume	Water Vol.	Template DNA OLD	Templa DNA	Total volume	Control	Ann Tem l C	GEL Experime Date	Band	Slid ID	Lane	Core Informatic
347	3-Jan-06	128	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	2	Core ID# 8 Secti
348	3-Jan-06	128	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	3	Core ID# 8 Secti
349	3-Jan-06	129	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	4	Core ID# 8 Secti
350	3-Jan-06	129	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	5	Core ID# 8 Secti
351	3-Jan-06	130	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	6	Core ID# 8 Secti
352	3-Jan-06	130	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	7	Core ID# 8 Secti
353	3-Jan-06	131	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	8	Core ID# 9 Secti
354	3-Jan-06	131	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	9	Core ID# 9 Secti
355	3-Jan-06	132	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	10	Core ID# 9 Secti
356	3-Jan-06	132	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	11	Core ID# 9 Secti
357	3-Jan-06	133	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06		37	12	Core ID# 9 Secti
358	3-Jan-06	133	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	13	Core ID# 9 Secti
359	3-Jan-06	134	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	14	Core ID# 7 Secti
360	3-Jan-06	134	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	15	Core ID# 7 Secti
361	3-Jan-06	135	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06		37	16	Core ID# 7 Secti
362	3-Jan-06	135	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	17	Core ID# 7 Secti
363	3-Jan-06	136	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00	X	46	4-Jan-06		37	18	Blank
364	3-Jan-06	136	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00	X	46	4-Jan-06	X	37	19	Blank
365	3-Jan-06	68	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06		37	20	Core ID# 6 Secti
366	3-Jan-06	68	E8F	1.00	E533R	1.00	12.50	5.50		5	25.00		46	4-Jan-06	X	37	22	Core ID# 6 Secti
367	6-Jan-06	51	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	6-Jan-06		38	2	Core ID# 2 Secti
368	6-Jan-06	71	E8F	1.00	E533R	1.00	12.50	7.50		3	25.00		46	6-Jan-06		38	3	Core ID# 7 Secti
369	6-Jan-06	51	E8F	1.00	E533R	1.00	12.50	9.50		1	25.00		46	6-Jan-06	X	38	4	Core ID# 2 Secti
370	6-Jan-06	71	E8F	1.00	E533R	1.00	12.50	9.50		1	25.00		46	6-Jan-06	X	38	5	Core ID# 7 Secti
371																		
372																		
373																		
374																		
375																		
376											0.00							

SubTotal from 22 Sep to 1 Nov 00 = 1455.00

Appendix D: Running Gel and Lab Procedures

Running Gel

1. **Mix TAE solution**
   a. First step will require you make the **Tris-acetic acid-disodium EDTA** (TAE) solution which is what makes the DNA happy. This solution comes from WSU at a 50X concentration. We need to make a 1X concentration. The formula for this is

$$C_1 * V_1 = C_2 * V_2$$

Equation 1

   b. For this requirement, we would like to get 500mL of TAE at a concentration of 1X so we plug in the known values to get the volume of TAE @ 50X concentration and then subtract that from the overall 500mL to get the amount of distilled water.

$$C_1 = 1X$$
$$V_1 = 1000mL$$
$$C_2 = 50X$$
$$V_2 = ?$$

   c. We plug in known values and solve for V_2:

$$\frac{(1X) * (1000mL)}{(50X)} = 20mL = V_2$$

   d. We then subtract the V2 from the overall solution that we want to make and that gives us the amount of Distilled water that we need which to make a 1X solution of 1000mL. This means we need [980mL of DI] and [20mL of TAE 50X] .

2. **Making of Agarose Gel**
   a. We need to make an Agarose solution for the gel bed.
      i. Following is an example for an .8% Agrose solution. You need to use the following equation to determine the proper quantites for your mixture.
         1. L = is the volume of TAE 1X in mL
         2. % = is the percent of Agarose solution you desire
         3. W = is the mass in grams of Agarose powder.

$$L * \% = W$$

Equation 2

   b. Following are examples of needed ingredients for a .9% gel
      i. Small Gel - We take 0.63g of Agarose into 70mL of our TAE 1X solution
      ii. Big Gel - We take 0.9g of Agarose into 100mL of our TAE 1X solution

c. Table 1 shows a quick summary of the different Agarose needed for the different gel sizes and the different percentages of Agorose. The greater the Agarose solution, the slower the PCR samples seem to travel.

		Small Gel	Large Gel
TAE 1X Solution (ml)		70	100
Agarose (g)	0.8%	0.56	0.8
	0.9%	0.63	0.9
	1.0%	0.7	1

Table 1

d. Mix and place into microwave. (Do not heat up TAE X1 solution before you add the Agarose powder, because it will coagulate and not mix thoroughly.
e. Set microwave for about 1.5 minutes to bring solution to boil. Check at about 1 minute to see if Agarose is completely dissolved.

What is a Gel?

i. You may be wondering what exactly a gel is, and what it has to do with agarose. Let's find out by "making" a gel. Purified agarose is in powdered form, and is insoluble in water (or buffer) at room temperature. But it dissolves in boiling water. When it starts to cool, it undergoes what is known as **polymerization**. Rather than staying dissolved in the water or coming out of solution, the sugar polymers crosslink with each other, causing the solution to "gel" into a semi-solid matrix much like "Jello" only more firm. The more agarose is dissolved in the boiling water, the firmer the gel will be. While the solution is still hot, we pour it into a mold called a "casting tray" so it will assume the shape we want as it polymerizes (otherwise it will just solidify in the bottom of the flask wasting the expensive agarose). (http://www.life.uiuc.edu/molbio/geldigest/electro.html)

f. Once Agarose is dissolved remove from microwave and add $70\mu L$ of ETBR 1000X and swirl

i. **General Information:** Ethidium Bromide (EtBr) is a commonly used stain for the visualization of nucleic acids in agrose gels. It is widely used by scientists due to its high sensitivity, rapid staining and very inexpensive price. While it is not specifically regulated as a hazardous waste, the mutagenic properties may present a hazard if it is not managed properly in the laboratory.
Safer Alternative: There is now a safer, more convenient and sensitive alternative to EtBr. Please go to the Molecular Probes website for additional information.

ii. **Personal Protection:** When handling EtBr always wear a lab coat, nitrile gloves, and chemical splash goggles. Proper skin and eye protection are needed when a ultraviolet (UV) light source is used while working with EtBr. Avoid exposing unprotected skin and eyes to intense UV sources. Wear a face shield if UV source is pointing upwards. When working with a UV source for a long time, wrap up lab coat sleeves with tape or other means where the wrist could be exposed.

iii. **Disposal of EtBr**: Electrophoresis Gels: Trace amounts of EtBr (less than 0.1%) in electrophoresis gels do not pose a serious hazard so they can be discarded in the trash if properly bagged and secured. If the gels contain more than 0.1% EtBr they should be placed in an appropriate container for hazardous waste

disposal. Environmental Health and Safety (EH&S) has a variety of containers that are available to collect and dispose of gels.

g. Place comb in gel box
h. Add the 70mL of solution to the Gel Bed and allow hardening approximately 30 minutes.
    i. Do the finger test
i. Once gel is hardened, remove comb from gel bed
j. Extract gel bed from gel box and rotate 90 degrees so that wells formed by the comb are opposite the red (Pos)leads.
k. Fill Gel box with 1X TAE until both sides of gel box overflow and cover the hardened gel.

3. Prepare PCR Samples for Gel

a. Take PCR sample and remove $3\mu L$ into a autoclaved eppendorf tube
b. 17μL of distilled water
c. $4\mu L$ of 6X buffer (blue, does not have to be refrigerated)

4. Load GEL DNA into Wells

a. Get pipet and set for $6\mu L$ of 1kb DNA Ladder
    i. Add this into the 1st well (Toward the Black (-) Lead)
b. Add $24\mu L$ of PCR samples for Gel prepared in step 3
    i. The $24\mu L$ is because that is the volume of sample created. There is not a set amount required for the wells. Bottom line is that the entire PCR sample prepared in step 3 needs to be injected into each well.
c. Attach colored leads to matching receptors on Gel box and power source
d. Turn on power source and allow to run until there is a clear separation (Approximatily ½ hour @ 120 v)

5. Imaging of GEL in Gel Logic 200

a. Log on to computer
    i. Login ID: user
    ii. Password: user
b. Controls on Imaging System
    i. Aperture opening
    ii. Zoom
    iii. Focus

2/9/04: Cheaper, Faster DNA Electrophoresis Possible with Common Cleanser

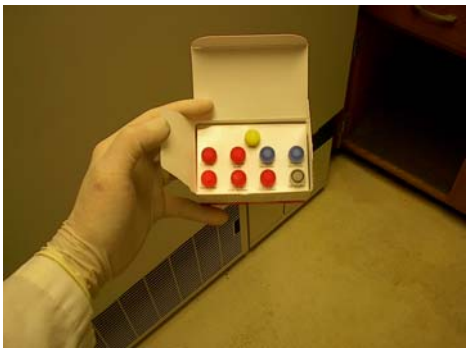
Johns Hopkins Kimmel Cancer Center scientists have found that substituting a simple, inexpensive bleach solution for more complex tools makes a DNA separation technique called electrophoresis five times faster and less costly. In the February issue of BioTechniques, the scientists report that using sodium boric acid, instead of time honored stand-bys Tris-acetic acid-disodium EDTA (TAE) and Tris-boric acid-disodium EDTA (TBE), in DNA electrophoresis may speed genetic discoveries. While TAE and TBE work well for protein electrophoresis, which uses molecules' different charges to separate them, the solutions aren't ideal for separating DNA. "In fact, TBE and TAE essentially short-circuit DNA gels by creating too much current and heat," says Scott Kern, MD, professor of oncology and pathology at the Johns Hopkins Kimmel Cancer Center.

Kern and postdoctoral fellow Jonathan Brody conducted experiments that showed that TBE and TAE provide only some buffering -- which they say isn't important anyway for DNA separation -- and too much conductivity. By trial and error, sodium boric acid turned out to be a good substitute. "It has great resolution at high voltages," says Brody. "I can now run a gel in 15 minutes using sodium borate as opposed to an hour and half with TBE or TAE."

CLONING

Setting up the TOPO Cloning reaction (Page 5)

1) Things you will need:
    i) Fresh PCR Product form PCR pool
    ii) TOPO TA Cloning Instruction Manual (Page 5)
    iii) TOPO TA Cloning Kit
        (1) Salt solution
        (2) Sterile water
        (3) TOPO Vector



Reagent	Chemically Competent E. coli (μl)
Fresh PCR product	1
Salt Solution	1
Sterile Water	3
TOPO Vector	1
Total Volume =	6

Table 2

2) Mix Reaction gently and incubate for 20 minutes at room temp (22-23° C)
    i) Note: This can be stored over night at -20°C

One Shot Chemical Transformation (Page 10)

1) Things you will need:
    i) One Shot Chemically Competent E. Coli Kit
    ii) Vial of One shot chemically competent E. Coli (Thaw on ice approx 30 min)
    iii) TOPO Cloning reaction from section I
    iv) Pre-warmed selected plates to 37° C (3 plates per PCR template pool)
    v) S.O.C. medium (room temperature)
    vi) 40mg/ml X-gal
    vii) Plate Spreader

viii) Bucket with Ice
ix) 42° C water bath
x) 37° C shaking incubator
xi) 37° C non-shaking incubator

2) Add 2 µl of TOPO Cloning reaction from step 1 to One Shot competent *E. Coli* vial

i) Do not mix by pipetting
3) Incubate on Ice for 30 minutes

4) Heat Shock the cells for 30 Seconds at 42° C
i) No shaking
ii) Normally done in the "Pierision Water Bath" at WSU

5) Immediately transfer the tubes to ice
    i) The ice can be taken from the ice machine near the autoclave

6) Add 250 ul of S.O.C. at room temperature.
7) Cap tube and shake horizontally (200 rpm) at (37° C) for 1 hour (this makes your transformation stock for the growing of colonies)

8) Get ready Pre-warm selected plates to (37° C) for 30 min
9) Spread 40µl of 40mg/ml X-gal on each plate
    i) Caution: X-gal stock solution contains Dimethylformide
    ii) X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) turns blue when incubated in the presence of b-glactosidase. This gene is on several of the cloning plasmids (especially, on the pUC series and lGT11 vectors). When an inserted piece of DNA is placed in the correct restriction site, the lacZ gene is interrupted and the colony does not turn the media blue (colony we want). Be sure to run controls.

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

Formula = $C_{14}H_{15}Br\ Cl\ N\ O_6$

10) Spread 50µl of transformation stock on a plate

11) Repeat last two steps on two other plates
12) Incubate over night at 37° C (12-18hrs)
13) Invert plates upside down to prevent condensation from dripping on colonies
14) Storage
    i) Bacteria can be stored several weeks on agar plates at 4°C if plates are wrapped in parafilm and stored inverted.
    ii) Medium-term storage in stab cultures
        (a) Inoculate a small vial containing 2-3 ml 1.5% agar/LB with a sterile, straight wire which was dipped into an overnight culture.
        (b) Grow stab culture o.n. at 37°C with the lid loose.
        (c) Tighten lid, wrap in parafilm, and store in dark at room temp.
    iii) Long. term storage at -70°C in 15% glycerol
        (a) Mix 150 µl sterile glycerol and 850 ul overnight culture
        (b) Store cultures at -20°C for few years or at -70°C for many years.

Culture Colonies

(This is found on page 12 of the TOPO TA Cloning Instruction Manual)

1) Things you will need:
    i) Scrapper
    ii) Burner
    iii) Cultured plates with colonies
    iv) Autoclaved glass tubes and cap (One tube for each colony scraped off plate)

    v) 37° C shaking incubator
2) Label each glass vial
3) Remove cap on glass vial
4) Burn mouth of glass vial
5) Fill each autoclaved glass tube with 2ml of LB containing 50 μg/ml of ampicillin
6) Re-burn glass vial and cap
7) Take your scraper and burn off in burner
8) Cool scraper by touching gel
9) Scrape off colony
10) Remove
11) Inoculate one glass vial filled with LB and ampicillin
12) Place glass vials in 37° C shaking incubator over night

Isolate and Purify Plasmid DNA

(This is found in the QIAGEN QIAprep Miniprep Handbook and statrs on page 22)

Things you will need:

QIAGEN QIAprep Miniprep Handbook Starts on page 22

1. Bacterial Media

LB Medium

10 g Bacto-tryptone
5 g Bacto-yeast extract
10 g NaCl
-dissolve in ~900 ml H_20
-adjust pH to 7.5 w/NaOH
-q.s. to 1L with H_2O

NZCYM

10 g NZ amine
5 g NaCl
5 g yeast extract
1 g casamino acids
2 g $MgSO_4 \cdot 7H_20$
-dissolve in ~900 ml
-adjust to pH 7.5 w/NaOH
-q.s. to 1L with H_2O

Media should be autoclaved minimum of 20 mins. on liquid cycle.

Plates and Top Agarose

Plates of 1.5% agar/LB medium are commonly used to grow bacteria. Antibiotics can be included in the medium, to use for specific selection procedures.

Preparation of 1.5% agar/LB medium plates:

i. Include 15 g. agar per liter medium before autoclaving. Agar will not dissolve until medium is autoclaved.

ii. To include antibiotics cool the autoclaved medium to ~55°C (a temp at which flask containing medium is cool enough to be held in hand, yet hot enough so it remains liquid). Add appropriate amount of a sterile antibiotic solution to achieve desired conc. Antibiotic solutions are sterilized by filtration through 0.22 μm filter (see CSH manual p.444 for making stocks).
Working concs.: Amp. = 50 μg/ml.
Tet. = 15-25 μg/ml

iii. After agar has cooled to ~55°C plates may be poured. This is done on the bench, using flame to keep media bottle sterile. Approx. 40-50 plates can be poured from 1 liter (10 cm sterile petri dishes). Flame to remove bubbles.

Top agarose - used in plating bacteriophage g-infected bacteria.
Preparation - 0.7% agarose/LB medium
Include 7 g agarose/liter medium before autoclaving. Store in 100-250 ml aliquots and melt in microwave before using.

Safety (MSDS) data for dimethylformamide

General

Synonyms: N,N-dimethylformamide, dimethyl formamide, N-formyldimethylamine, DMF, U-4224, DMFA, NSC 5356
Molecular formula: $HCON(CH_3)_2$
CAS No: 68-12-2
EC No: 200-679-5

Physical data

Appearance: colourless liquid with slight ammonia odour
Melting point: -61 C
Boiling point: 153 C
Vapour density: 2.5
Vapour pressure: 2.6 mm Hg at 20 C
Specific gravity: 0.95
Flash point: 58 C
Explosion limits: 2.2% - 15.2%
Autoignition temperature: 445 C

Stability

Stable. Incompatible with strong oxidising agents, halogenated hydrocarbons, chloroformates, active halogen compounds, strong acids, strong reducing agents, rubber, leather.

Appendix E: Loading Gel Specification Sheet

invitrogen

BlueJuice™ Gel Loading Buffer

Cat. No. 10816-015 **Size: 3 × 1 ml**
Store at +4°C

Description

BlueJuice™ Gel Loading Buffer (10X) is a liquid buffer containing all of the components necessary for easy loading and tracking of DNA samples in agarose or native polyacrylamide gels.

BlueJuice™ Gel Loading Buffer (10X)

65% (w/v) Sucrose
10 mM Tris-HCl (pH 7.5)
10 mM EDTA
0.3% (w/v) Bromophenol Blue

The product is stable for 6 months when stored properly.

Protocol

The recommended concentration of this buffer for use with all DNA samples run on agarose gels is 2X (one part buffer plus four parts sample). For acrylamide gels, the recommended concentration is 1X (one part buffer plus nine parts sample).

Note: Concentrations higher than 1X applied to native polyacrylamide gels may cause the bands to "smile" slightly. Any concentration may be used in agarose gels without affecting band appearance (except for bands that may be obscured by the bromophenol blue tracking dye).

Part No. 10816015.pps Rev. Date: 19 Sep 2005

Appendix F: Polymerase Chain Reaction Pooling Log

PCR Pool ID	PCR ID	Core ID	Section	DNA Template ID	Date	Note
252P	251	4	3	33	9-Dec-05	
252P	252	4	3	33	9-Dec-05	
254P	253	4	1	38	9-Dec-05	
254P	254	4	1	38	9-Dec-05	
256P	255	4	2	40	9-Dec-05	
256P	256	4	2	40	9-Dec-05	
258P	246	2	3	54	9-Dec-05	
258P	258	2	3	54	9-Dec-05	
262P	232	1	1	61		
262P	262	1	1	61		
265P	226	1	3	63		
265P	265	1	3	63		
266P	235	5	1	64	9-Dec-05	
266P	266	5	1	64	9-Dec-05	
267P	228	5	2	65	9-Dec-05	
267P	267	5	2	65	9-Dec-05	
270P	238	6	1	67	9-Dec-05	
270P	270	6	1	67	9-Dec-05	
~~271P~~	~~239~~	~~6~~	~~2~~	~~68~~	9-Dec-05	
~~271P~~	~~271~~	~~6~~	~~2~~	~~68~~	9-Dec-05	
272P	240	6	3	69		
272P	272	6	3	69		
278P	264	1	2	62		
278P	278	1	2	62		
279P	259	2	1	58		
279P	279	2	1	58		
280P	260	2	2	59		
280P	280	2	2	59		
283P	275	7	3	72		
283P	283	7	3	72		
285P	284	5	3	19	9-Dec-05	
285P	285	5	3	19	9-Dec-05	
317P	316	9	3	114	11-Jan-06	
317P	317	9	3	114	11-Jan-06	
323P	322	BLK			11-Jan-06	Slide 32
323P	323	BLK			11-Jan-06	Slide 32
348P	347	8	1	41	6-Jan-06	
348P	348	8	1	41	6-Jan-06	
350P	349	8	2	43	6-Jan-06	
350P	350	8	2	43	6-Jan-06	
352P	351	8	3	46	6-Jan-06	
352P	352	8	3	46	6-Jan-06	
354P	353	9	1	47	6-Jan-06	
354P	354	9	1	47	6-Jan-06	
356P	355	9	2	49	6-Jan-06	
356P	356	9	2	49	6-Jan-06	
360P	273	7	1	70	6-Jan-06	
360P	360	7	1	23	6-Jan-06	
364P	363	BLK			6-Jan-06	Slide 37
364P	364	BLK			6-Jan-06	Slide 37
366P	343	6	2	68	6-Jan-06	
366P	366	6	2	68	6-Jan-06	
370P	362	7	2	135	11-Jan-06	
370P	370	7	2	71	11-Jan-06	

Appendix G: Invitrogen TOPO TA Cloning™ Procedures

TOPO TA Cloning®

Version R
8 April 2004
25-0184

TOPO TA Cloning®

Five-minute cloning of *Taq* polymerase-amplified PCR products

Catalog nos. K4500-01, K4500-40, K4510-20, K4520-01, K4520-40, K4550-01, K4550-40, K4560-01, K4560-40 (pCR®2.1-TOPO®)

Catalog nos. K4600-01, K4600-40, K4610-20, K4620-01, K4620-40, K4650-01, K4650-40, K4660-01, K4660-40 (pCR®II-TOPO®)

A Limited Label License covers this product (see Purchaser Notification). By use of this product, you accept the terms and conditions of the Limited Label License.

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Kit Contents and Storage

Shipping and Storage

TOPO TA Cloning® Kits are shipped on dry ice. Each kit contains a box with TOPO TA Cloning® reagents (Box 1) and a box with One Shot® Chemically Competent or Electrocomp™ cells (Box 2).

Store Box 1 at -20°C and Box 2 at -80°C.

Types of TOPO TA Cloning® Kits

TOPO TA Cloning® Kits are available with either pCR®2.1-TOPO® or pCR®II-TOPO® and either DH5α™-T1^R, Mach1™-T1^R, TOP10, or TOP10F´ One Shot® Chemically Competent cells or TOP10 One Shot® Electrocomp™ cells (see page vii for the genotypes of the strains).

Product	Reactions	One Shot® Cells	Type of Cells	Catalog no.
TOPO TA Cloning® Kit (containing pCR®2.1-TOPO®)	20	TOP10	chem. competent	K4500-01
	40	TOP10	chem. competent	K4500-40
	20	Mach1™-T1^R	chem. competent	K4510-20
	20	DH5α™-T1^R	chem. competent	K4520-01
	40	DH5α™-T1^R	chem. competent	K4520-40
	20	TOP10F´	chem. competent	K4550-01
	40	TOP10F´	chem. competent	K4550-40
	20	TOP10	electrocompetent	K4560-01
	40	TOP10	electrocompetent	K4560-40
TOPO TA Cloning® Kit Dual Promoter (containing pCR®II-TOPO®)	20	TOP10	chem. competent	K4600-01
	40	TOP10	chem. competent	K4600-40
	20	Mach1™-T1^R	chem. competent	K4610-20
	20	DH5α™-T1^R	chem. competent	K4620-01
	40	DH5α™-T1^R	chem. competent	K4620-40
	20	TOP10F´	chem. competent	K4650-01
	40	TOP10F´	chem. competent	K4650-40
	20	TOP10	electrocompetent	K4660-01
	40	TOP10	electrocompetent	K4660-40

continued on next page

Kit Contents and Storage, continued

TOPO TA Cloning® Reagents

TOPO TA Cloning® reagents (Box 1) are listed below. Note that the user must supply Taq polymerase. Store Box 1 at -20°C.

Item	Concentration	Amount
pCR®2.1-TOPO® or pCR®II-TOPO®	10 ng/µl plasmid DNA in: 50% glycerol 50 mM Tris-HCl, pH 7.4 (at 25°C) 1 mM EDTA 1 mM DTT 0.1% Triton X-100 100 µg/ml BSA phenol red	20 µl
10X PCR Buffer	100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100 µl
Salt Solution	1.2 M NaCl 0.06 M $MgCl_2$	50 µl
dNTP Mix	12.5 mM dATP; 12.5 mM dCTP 12.5 mM dGTP; 12.5 mM dTTP neutralized at pH 8.0 in water	10 µl
M13 Forward (-20) Primer	0.1 µg/µl in TE Buffer	20 µl
M13 Reverse Primer	0.1 µg/µl in TE Buffer	20 µl
Control Template	0.1 µg/µl in TE Buffer	10 µl
Control PCR Primers	0.1 µg/µl each in TE Buffer	10 µl
Sterile Water	–	1 ml

Sequence of Primers

The table below describes the sequence and pmoles supplied of the sequencing primers included in this kit.

Primer	Sequence	pMoles Supplied
M13 Forward (-20)	5´-GTAAAACGACGGCCAG-3´	407
M13 Reverse	5´-CAGGAAACAGCTATGAC-3´	385

continued on next page

One Shot® Reagents

The table below describes the items included in each One Shot® competent cell kit. Store at -80°C.

Item	Composition	Amount
S.O.C. Medium (may be stored at +4°C or room temperature)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
TOP10, Mach1™-T1^R, DH5α™-T1^R, or TOP10F´ cells **OR** TOP10 cells	Chemically Competent Electrocomp™	21 x 50 µl
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotypes of *E. coli* Strains

DH5α™-T1^R: Use this strain for general cloning and blue/white screening without IPTG. Strain is resistant to T1 bacteriophage.

F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A (confers resistance to phage T1)

Mach1™-T1^R: Use this strain for general cloning and blue/white screening without IPTG. Strain is resistant to T1 bacteriophage.

F- ϕ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R(r_k^-, m_k^+) Δ*rec*A1398 *end*A1 *ton*A (confers resistance to phage T1)

TOP10: Use this strain for general cloning and blue/white screening without IPTG.

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

TOP10F´: This strain overexpresses the Lac repressor (*lac*I^q gene). For blue/white screening, you will need to add IPTG to the plates to obtain expression from the *lac* promoter. This strain contains the F episome and can be used for single-strand rescue of plasmid DNA containing an f1 origin.

F´ {*lac*I^q Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Information for Non-U.S. Customers Using Mach1™-T1^R Cells

The parental strain of Mach1™-T1^R *E. coli* is the non-K-12, wild-type W strain (ATCC #9637, S. A. Waksman). Although the parental strain is generally classified as Biosafety Level 1 (BL-1), we recommend that you consult the safety department of your institution to verify the Biosafety Level.

Accessory Products

Additional Products

The table below lists additional products that may be used with TOPO® TA Cloning Kits. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 24).

Item	Amount	Catalog no.
Taq DNA Polymerase, Native	100 units	18038-018
	500 units	18038-042
Taq DNA Polymerase, Recombinant	100 units	10342-053
	500 units	10342-020
Platinum® *Taq* DNA Polymerase High Fidelity	100 units	11304-011
One Shot® TOP10 Chemically Competent *E. coli*	10 reactions	C4040-10
	20 reactions	C4040-03
	40 reactions	C4040-06
One Shot® TOP10 Electrocompetent *E. coli*	10 reactions	C4040-50
	20 reactions	C4040-52
One Shot® Mach1™-T1^R Chemically Competent *E. coli*	20 reactions	C8620-03
One Shot® MAX Efficiency® DH5α-T1^R Chemically Competent *E. coli*	20 reactions	12297-016
One Shot® TOP10F´ Chemically Competent *E. coli*	20 reactions	C3030-03
	40 reactions	C3030-06
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
Ampicillin	200 mg	11593-019
Kanamycin	5 g	11815-024
	25 g	11815-032
	100 ml (10 mg/ml)	18160-054
X-gal	100 mg	15520-034
	1 g	15520-018
IPTG	1 g	15529-019
S.O.C. Medium	10 x 10 ml	15544-034

Methods

Overview

Introduction

TOPO TA Cloning®* provides a highly efficient, 5-minute, one-step cloning strategy ("TOPO® Cloning") for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required.

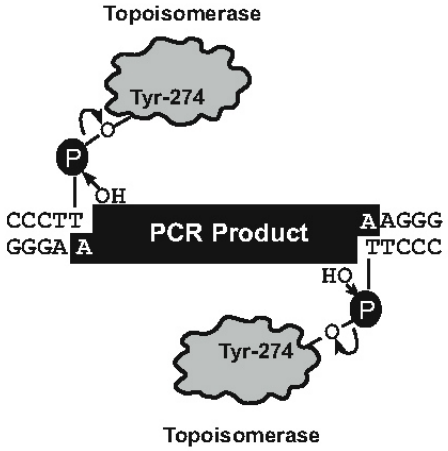
How It Works

The plasmid vector (pCR®II-TOPO® or pCR®2.1-TOPO®) is supplied linearized with:

- Single 3´-thymidine (T) overhangs for TA Cloning®
- Topoisomerase I covalently bound to the vector (referred to as "activated" vector)

Taq polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′-CCCTT in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994).



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Overview, continued

Experimental Outline

- Produce Your PCR Product
- Set Up TOPO® Cloning Reaction (Mix Together PCR Product and TOPO® Vector)
- Incubate 5 Minutes at Room Temperature
- Transform TOPO® Cloning Reaction into One Shot® Competent Cells
- Select and Analyze 10 White or Light Blue Colonies for Insert

Producing PCR Products

Introduction

It is important to properly design your PCR primers to ensure that you obtain the product you need for your studies. Once you have decided on a PCR strategy and have synthesized the primers, you are ready to produce your PCR product. **Remember that your PCR product will have single 3´ adenine overhangs.**

Note

Do not add 5´ phosphates to your primers for PCR. The PCR product synthesized will not ligate into pCR®2.1-TOPO® or pCR®II-TOPO®.

Materials Supplied by the User

You will need the following reagents and equipment.

- *Taq* polymerase
- Thermocycler
- DNA template and primers for PCR product

Polymerase Mixtures

If you wish to use a mixture containing Taq polymerase and a proofreading polymerase, Taq must be used in excess of a 10:1 ratio to ensure the presence of 3´ A-overhangs on the PCR product.

If you use polymerase mixtures that do not have enough Taq polymerase or a proofreading polymerase only, you can add 3′ A-overhangs using the method on page 22.

Producing PCR Products

1. Set up the following 50 µl PCR reaction. Use less DNA if you are using plasmid DNA as a template and more DNA if you are using genomic DNA as a template. Use the cycling parameters suitable for your primers and template. Be sure to include a 7 to 30 minute extension at 72°C after the last cycle to ensure that all PCR products are full length and 3´ adenylated.

DNA Template	10-100 ng
10X PCR Buffer	5 µl
50 mM dNTPs	0.5 µl
Primers (100-200 ng each)	1 µM each
Sterile water	add to a final volume of 49 µl
Taq Polymerase (1 unit/µl)	1 µl
Total Volume	50 µl

2. Check the PCR product by agarose gel electrophoresis. You should see a single, discrete band. If you do not see a single band, refer to the **Note** below.

Note

If you do not obtain a single, discrete band from your PCR, you may gel-purify your fragment before using the TOPO TA Cloning® Kit (see page 20). Take special care to avoid sources of nuclease contamination. Alternatively, you may optimize your PCR to eliminate multiple bands and smearing (Innis *et al.*, 1990). The PCR Optimizer™ Kit (Catalog no. K1220-01) incorporates many of the recommendations found in this reference. For more information, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 24).

Setting Up the TOPO® Cloning Reaction

Introduction

Once you have produced the desired PCR product, you are ready to TOPO® Clone it into the pCR®2.1-TOPO® or pCR®II-TOPO® vector and transform the recombinant vector into competent *E. coli*. It is important to have everything you need set up and ready to use to ensure that you obtain the best possible results. We suggest that you read this section and the sections detailing transformation of competent cells (pages 6-10) before beginning. If this is the first time you have TOPO® Cloned, perform the control reactions on pages 17-18 in parallel with your samples.

Note

Recent experiments at Invitrogen demonstrate that inclusion of salt (200 mM NaCl; 10 mM $MgCl_2$) in the TOPO® Cloning reaction increases the number of transformants 2- to 3-fold. We have also observed that in the presence of salt, incubation times of greater than 5 minutes can also increase the number of transformants. This is in contrast to earlier experiments **without salt** where the number of transformants decreases as the incubation time increases beyond 5 minutes.

Inclusion of salt allows for longer incubation times because it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. The result is more intact molecules leading to higher transformation efficiencies.

Important

Because of the above results, we recommend adding salt to the TOPO® Cloning reaction. A stock salt solution is provided in the kit for this purpose. **Note that the amount of salt added to the TOPO® Cloning reaction varies depending on whether you plan to transform chemically competent cells or electrocompetent cells (see below).** For this reason two different TOPO® Cloning reactions are provided to help you obtain the best possible results. Read the following information carefully.

Transforming Chemically Competent *E. coli*

For TOPO® Cloning and transformation into chemically competent *E. coli*, adding sodium chloride and magnesium chloride to a final concentration of 200 mM NaCl, 10 mM $MgCl_2$ in the TOPO® Cloning reaction increases the number of colonies over time. A Salt Solution (1.2 M NaCl; 0.06 M $MgCl_2$) is provided to adjust the TOPO® Cloning reaction to the recommended concentration of NaCl and $MgCl_2$.

Transforming Electrocompetent *E. coli*

For TOPO® Cloning and transformation of electrocompetent *E. coli*, salt must also be included in the TOPO® Cloning reaction, but the amount of salt **must be reduced** to 50 mM NaCl, 2.5 mM $MgCl_2$ to prevent arcing. The Salt Solution is diluted 4-fold to prepare a 300 mM NaCl, 15 mM $MgCl_2$ solution for convenient addition to the TOPO® Cloning reaction (see next page).

continued on next page

Setting Up the TOPO® Cloning Reaction, continued

Setting Up the TOPO® Cloning Reaction

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into either chemically competent or electrocompetent TOP10 or chemically competent DH5α™-T1^R, Mach1™-T1^R, or TOP10F′ One Shot® *E. coli*. Additional information on optimizing the TOPO® Cloning reaction for your needs can be found on page 14.

Note: The red color of the TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	–
Dilute Salt Solution	–	1 µl
Sterile Water	add to a total volume of 5 µl	add to a total volume of 5 µl
TOPO® vector	1 µl	1 µl
Final Volume	**6 µl**	**6 µl**

* Store all reagents at -20°C when finished. Salt solutions and water can be stored at room temperature or +4°C.

Performing the TOPO® Cloning Reaction

1. Mix reaction gently and incubate for **5 minutes** at room temperature (22-23°C).

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (>1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.

2. Place the reaction on ice and proceed to **General Guidelines for Transforming Competent Cells**, next page.

   Note: You may store the TOPO® Cloning reaction at -20°C overnight.

General Guidelines for Transforming Competent Cells

Introduction

Once you have performed the TOPO® Cloning reaction, you will transform your pCR®2.1-TOPO® or pCR®II-TOPO® construct into the competent *E. coli* provided with your kit. General guidelines for transformation are provided below. For transformation protocols, refer to the section entitled **Transforming One Shot® Mach1™-T1^R Competent Cells** (pages 7-8) or **Transforming One Shot® DH5α™-T1^R, TOP10, and TOP10F´ Competent Cells** (pages 9-11) depending on the competent *E. coli* you wish to transform.

Selecting a One Shot® Chemical Transformation Protocol

Two protocols are provided to transform One Shot® chemically competent *E. coli*. Consider the following factors when choosing the protocol that best suits your needs.

If you wish to...	Then use the...
maximize the number of transformants	regular chemical transformation protocol
clone large PCR products (>1000 bp)	
use kanamycin as the selective agent (see Important note below)	
obtain transformants as quickly as possible	rapid chemical transformation protocol

Important

If you will be using kanamycin as the selective agent for chemical transformation, use the regular chemical transformation protocol. The rapid chemical transformation protocol is only suitable for transformations using ampicillin selection.

If you use a plasmid template for your PCR that carries either the ampicillin or kanamycin resistance marker, we recommend that you use the other selection agent to select for transformants. For example, if the plasmid template contains the ampicillin resistance marker, then use kanamycin to select for transformants. The template is carried over into the TOPO® Cloning and transformation reactions, resulting in transformants that are ampicillin resistant and white, but are not the desired construct.

Transforming One Shot® Mach1™-T1^R Competent Cells

Introduction

Protocols to transform One Shot® Mach1™-T1^R chemically competent *E. coli* are provided below. If are transforming cells other than Mach1™-T1^R cells, refer to the section entitled **Transforming One Shot® DH5α™-T1^R, TOP10, and TOP10F´ Competent Cells** (pages 9-11).

Note

The Mach1™-T1^R strain allows you to visualize colonies 8 hours after plating on ampicillin selective plates. If you are using kanamycin selection, you will need to incubate plates overnight in order to visualize colonies.

With the Mach1™-T1^R strain, you may also prepare plasmid DNA 4 hours after inoculating a single, overnight-grown colony. Note that you will get sufficient growth of transformed cells within 4 hours in either ampicillin or kanamycin selective media.

Materials Supplied by the User

In addition to general microbiological supplies (*e.g.* plates, spreaders), you will need the following reagents and equipment.

- TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2 (page 5)
- S.O.C. medium (included with the kit)
- LB plates containing 50 µg/ml ampicillin or 50 µg/ml kanamycin
- 40 mg/ml X-gal in dimethylformamide (DMF)
- 42°C water bath
- 37°C shaking and non-shaking incubator

Preparing for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes (see Important note below).
- Spread 40 µl of 40 mg/ml X-gal on each LB plate and incubate at 37°C until ready for use.
- Thaw **on ice** 1 vial of One Shot® cells for each transformation.

Important

If you are performing the rapid chemical transformation protocol or if you wish to visualize colonies within 8 hours of plating, it is essential that you prewarm your LB plates containing 50-100 µg/ml ampicillin prior to spreading.

continued on next page

Transforming One Shot® Mach1™-T1^R Competent Cells, continued

One Shot® Chemical Transformation Protocol

For optimal growth of Mach1™-T1^R *E. coli* cells, it is essential that selective plates are prewarmed to 37°C prior to spreading.

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 µl from each transformation on a **prewarmed** selective plate. To ensure even spreading of small volumes, add 20 µl of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. Incubate plates at 37°C. If you are using ampicillin selection, visible colonies should appear within 8 hours, and blue/white screening can be performed after 12 hours. For kanamycin selection, incubate plates overnight.
9. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 white or light blue colonies for analysis (see Analyzing Positive Clones, page 12). Do not pick dark blue colonies.

Rapid One Shot® Chemical Transformation Protocol

An alternative protocol is provided below for rapid transformation of One Shot® Mach1™-T1^R cells. This protocol is **only** recommended for transformations using **ampicillin** selection. For more information on selecting a transformation protocol, refer to page 6.

Note: It is essential that LB plates containing ampicillin are prewarmed to 37°C prior to spreading.

1. Add **4 µl** of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 minutes.
3. Spread **50 µl** of cells on a prewarmed LB plate containing 50-100 µg/ml ampicillin and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 white or light blue colonies for analysis (see Analyzing Positive Clones, page 12). Do not pick dark blue colonies.

Transforming One Shot® DH5α™-T1^R, TOP10, and TOP10F´ Competent Cells

Introduction

Protocols to transform One Shot® DH5α™-T1^R, TOP10, and TOP10F´ competent *E. coli* are provided below. Both chemical transformation and electroporation protocols are provided. If you are transforming Mach1™-T1^R cells, refer to the section entitled **Transforming One Shot® Mach1™-T1^R Competent Cells** (pages 7-8).

Materials Supplied by the User

In addition to general microbiological supplies (*e.g.* plates, spreaders), you will need the following reagents and equipment.

- TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2 (page 5)
- S.O.C. medium (included with the kit)
- LB plates containing 50 µg/ml ampicillin or 50 µg/ml kanamycin
- 40 mg/ml X-gal in dimethylformamide (DMF)
- 100 mM IPTG in water (for use with TOP10F´)
- 15 ml snap-cap plastic culture tubes (sterile) (electroporation only)
- 42°C water bath or an electroporator and 0.1 or 0.2 cm cuvettes
- 37°C shaking and non-shaking incubator

Preparation for Transformation

For each transformation, you will need one vial of competent cells and two selective plates.

- Equilibrate a water bath to 42°C (for chemical transformation) or set up your electroporator.
- Warm the vial of S.O.C. medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes (see Important note below).
- Spread 40 µl of 40 mg/ml X-gal on each LB plate and incubate at 37°C until ready for use.
- For TOP10F´ cells, spread 40 µl of 100 mM IPTG in addition to X-gal on each LB plate and incubate at 37°C until ready for use. IPTG is required for blue/white screening.
- Thaw **on ice** 1 vial of One Shot® cells for each transformation.

Important

If you are performing the rapid chemical transformation protocol, it is essential that you prewarm your LB plates containing 50-100 µg/ml ampicillin prior to spreading.

continued on next page

Transforming One Shot® DH5α™-T1^R, TOP10, and TOP10F´ Competent Cells, continued

One Shot® Chemical Transformation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 to 30 minutes.

   Note: Longer incubations on ice do not seem to have any affect on transformation efficiency. The length of the incubation is at the user's discretion.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. medium We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 white or light blue colonies for analysis (see Analyzing Positive Clones, page 12). Do not pick dark blue colonies.

Rapid One Shot® Chemical Transformation Protocol

An alternative protocol is provided below for rapid transformation of One Shot® chemically competent *E. coli*. This protocol is **only** recommended for transformations using **ampicillin** selection. For more information on selecting a transformation protocol, refer to page 6.

Note: It is essential that LB plates containing ampicillin are prewarmed prior to spreading.

1. Add **4 µl** of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Incubate on ice for 5 minutes.
3. Spread **50 µl** of cells on a prewarmed LB plate containing 50-100 µg/ml ampicillin and incubate overnight at 37°C.
4. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 white or light blue colonies for analysis (see Analyzing Positive Clones, page 12). Do not pick dark blue colonies.

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Transforming One Shot® DH5α™-T1^R, TOP10, and TOP10F´ Competent Cells, continued

One Shot® Electroporation Protocol

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2, page 5 into a vial of One Shot® Electrocompetent *E. coli* and mix gently. **Do not mix by pipetting up and down**.
2. Carefully transfer solution to a 0.1 cm cuvette to avoid formation of bubbles.
3. Electroporate your samples using your own protocol and your electroporator.

   Note: If you have problems with arcing, see below.
4. Immediately add 250 µl of room temperature S.O.C. medium.
5. Transfer the solution to a 15 ml snap-cap tube (*e.g.* Falcon) and shake for at least 1 hour at 37°C to allow expression of the antibiotic resistance genes.
6. Spread 10-50 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
7. An efficient TOPO® Cloning reaction should produce several hundred colonies. Pick ~10 white or light blue colonies for analysis (see Analyzing Positive Clones, next page). Do not pick dark blue colonies.

Note

Addition of the Dilute Salt Solution in the TOPO® Cloning Reaction brings the final concentration of NaCl and $MgCl_2$ in the TOPO® Cloning reaction to 50 mM and 2.5 mM, respectively. To prevent arcing of your samples during electroporation, the volume of cells should be between 50 and 80 µl (0.1 cm cuvettes) or 100 to 200 µl (0.2 cm cuvettes).

If you experience arcing, try **one** of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

Analyzing Transformants

Analyzing Positive Clones

1. Take the 10 white or light blue colonies and culture them overnight in LB medium containing 50 µg/ml ampicillin or 50 µg/ml kanamycin.

   Note: If you transformed One Shot® Mach1™-T1^R competent *E. coli*, you may inoculate overnight-grown colonies and culture them for 4 hours in **prewarmed** LB medium containing 50 µg/ml ampicillin or 50 µg/ml kanamycin before isolating plasmid. For optimal results, we recommend inoculating as much of a single colony as possible.

2. Isolate plasmid DNA using your method of choice. If you need ultra-pure plasmid DNA for automated or manual sequencing, we recommend the S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01)or the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01).
3. Analyze the plasmids by restriction analysis to confirm the presence and correct orientation of the insert. Use a restriction enzyme or a combination of enzymes that cut once in the vector and once in the insert.

Sequencing

You may sequence your construct to confirm that your gene is cloned in the correct orientation. The M13 Forward (-20) and M13 Reverse primers are included to help you sequence your insert. Refer to the maps on page 15 (pCR®2.1-TOPO®) or page 16 (pCR®II-TOPO®) for sequence surrounding the TOPO TA Cloning® site. For the full sequence of either vector, refer to our Web site (www.invitrogen.com) or contact Technical Service (page 24).

Analyzing Transformants by PCR

You may wish to use PCR to directly analyze positive transformants. For PCR primers, use either the M13 Forward (-20) or the M13 Reverse primer and a primer that hybridizes within your insert. If you are using this technique for the first time, we recommend performing restriction analysis in parallel. Artifacts may be obtained because of mispriming or contaminating template. The protocol is provided below for your convenience. Other protocols are suitable.

Materials Needed

PCR SuperMix High Fidelity (Invitrogen, Catalog no. 10790-020)

Appropriate forward and reverse PCR primers (20 µM each)

Procedure

1. For each sample, aliquot 48 µl of PCR SuperMix High Fidelity into a 0.5 ml microcentrifuge tube. Add 1 µl each of the forward and reverse PCR primer.
2. Pick 10 colonies and resuspend them individually in 50 µl of the PCR cocktail from Step 1, above. Don't forget to make a patch plate to preserve the colonies for further analysis.
3. Incubate the reaction for 10 minutes at 94°C to lyse the cells and inactivate nucleases.
4. Amplify for 20 to 30 cycles.
5. For the final extension, incubate at 72°C for 10 minutes. Store at +4°C.
6. Visualize by agarose gel electrophoresis.

continued on next page

Analyzing Transformants, continued

Important

If you have problems obtaining transformants or the correct insert, perform the control reactions described on pages 17-18. These reactions will help you troubleshoot your experiment.

Long-Term Storage

Once you have identified the correct clone, be sure to prepare a glycerol stock for long term storage. We recommend that you store a stock of plasmid DNA at -20°C.

1. Streak the original colony out on LB plates containing 50 µg/ml ampicillin or 50 µg/ml kanamycin.
2. Isolate a single colony and inoculate into 1-2 ml of LB containing 50 µg/ml ampicillin or kanamycin.
3. Grow until culture reaches stationary phase.
4. Mix 0.85 ml of culture with 0.15 ml of sterile glycerol and transfer to a cryovial.
5. Store at -80°C.

Optimizing the TOPO® Cloning Reaction

Introduction

The information below will help you optimize the TOPO® Cloning reaction for your particular needs.

Faster Subcloning

The high efficiency of TOPO® Cloning technology allows you to streamline the cloning process. If you routinely clone PCR products and wish to speed up the process, consider the following:

- Incubate the TOPO® Cloning reaction for only 30 seconds instead of 5 minutes.

  You may not obtain the highest number of colonies, but with the high efficiency of TOPO® Cloning, most of the transformants will contain your insert.
- After adding 2 µl of the TOPO® Cloning reaction to chemically competent cells, incubate on ice for only 5 minutes.

  Increasing the incubation time to 30 minutes does not significantly improve transformation efficiency.

More Transformants

If you are TOPO® Cloning large PCR products, toxic genes, or cloning a pool of PCR products, you may need more transformants to obtain the clones you want. To increase the number of colonies:

- Incubate the salt-supplemented TOPO® Cloning reaction for 20 to 30 minutes instead of 5 minutes.

  Increasing the incubation time of the salt-supplemented TOPO® Cloning reaction allows more molecules to ligate, increasing the transformation efficiency. Addition of salt appears to prevent topoisomerase from rebinding and nicking the DNA after it has ligated the PCR product and dissociated from the DNA.

Cloning Dilute PCR Products

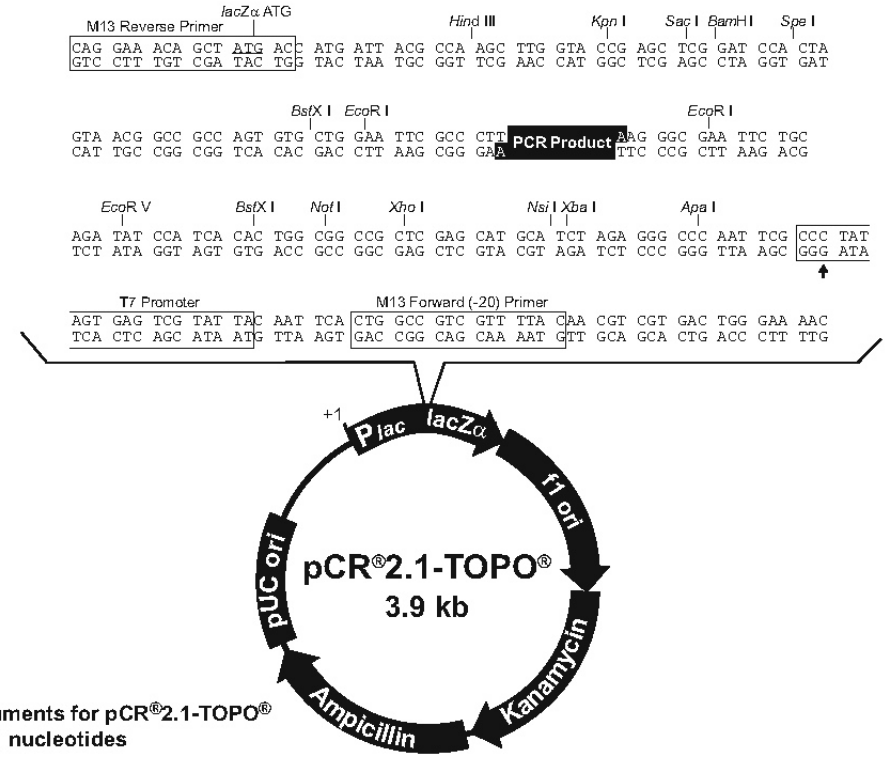
To clone dilute PCR products, you may:

- Increase the amount of the PCR product
- Incubate the TOPO® Cloning reaction for 20 to 30 minutes
- Concentrate the PCR product

Map of pCR®2.1-TOPO®

pCR®2.1-TOPO® Map

The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. **The complete sequence of pCR®2.1-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 24).**



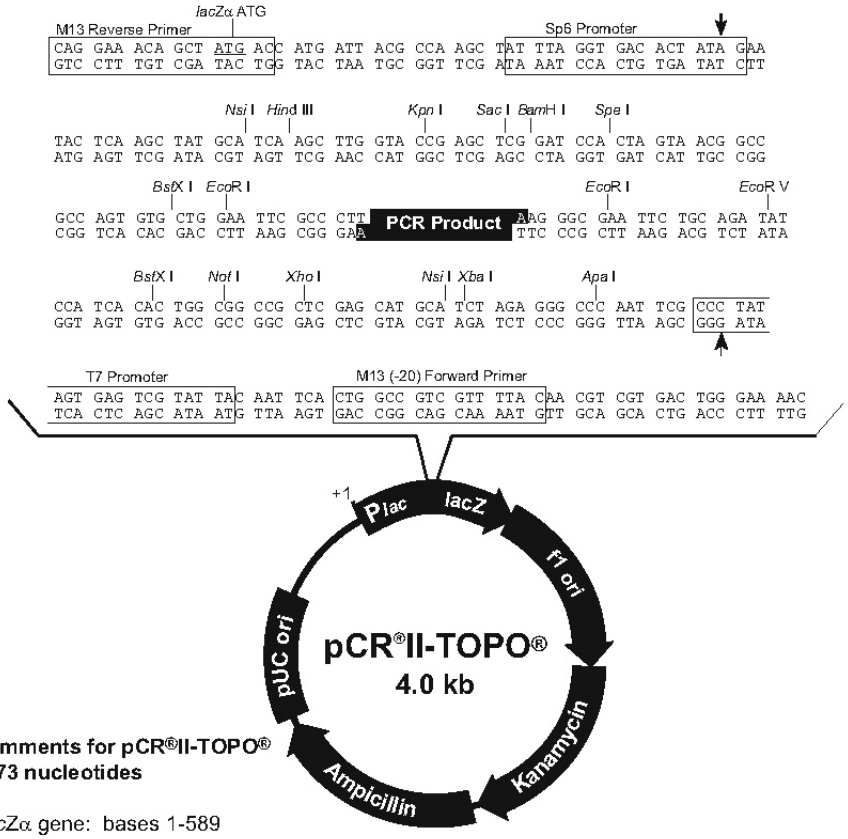
Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Map of pCR®II-TOPO®

pCR®II-TOPO® Map

The map below shows the features of pCR®II-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrows indicate the start of transcription for Sp6 and T7 polymerases. **The complete sequence of pCR®II-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 24).**



Comments for pCR®II-TOPO®
3973 nucleotides

LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851

Performing the Control Reactions

Introduction

We recommend performing the following control TOPO® Cloning reactions the first time you use the kit to help you evaluate your results. Performing the control reactions involves producing a control PCR product using the reagents included in the kit and using the PCR product directly in a TOPO® Cloning reaction.

Before Starting

For each transformation, prepare two LB plates containing 50 µg/ml kanamycin.

Note: Do not use plates containing ampicillin. The control template is a plasmid that encodes ampicillin resistance. This template is carried over into the TOPO® Cloning and transformation reactions. Transformants carrying this plasmid will also be ampicillin resistant and white, resulting in an apparent increase in TOPO® Cloning efficiency, but upon analysis, colonies do not contain the desired construct.

Producing Control PCR Product

1. To produce the 750 bp control PCR product, set up the following 50 µl PCR:

Control DNA Template (100 ng)	1 µl
10X PCR Buffer	5 µl
dNTP Mix	0.5 µl
Control PCR Primers (0.1 µg/ µl each)	1 µl
Sterile Water	41.5 µl
Taq Polymerase (1 unit/ µl)	1 µl
Total Volume	50 µl

2. Overlay with 70 µl (1 drop) of mineral oil.
3. Amplify using the following cycling parameters:

Step	Time	Temperature	Cycles
Initial Denaturation	2 minute	94°C	1X
Denaturation	1 minute	94°C	25X
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minutes	72°C	1X

4. Remove 10 µl from the reaction and analyze by agarose gel electrophoresis. A discrete 750 bp band should be visible. Proceed to the **Control TOPO® Cloning Reactions**, next page.

continued on next page

Control TOPO® Cloning Reactions

Using the control PCR product produced on the previous page and the TOPO® vector set up two 6 µl TOPO® Cloning reactions as described below.

1. Set up control TOPO® Cloning reactions:

Reagent	"Vector Only"	"Vector + PCR Insert"
Control PCR Product	–	1 µl
Sterile Water	4 µl	3 µl
Salt Solution or Dilute Salt Solution	1 µl	1 µl
TOPO® vector	1 µl	1 µl

2. Incubate at room temperature for **5 minutes** and place on ice.
3. Transform 2 µl of each reaction into separate vials of One Shot® competent cells (pages 6-10).
4. Spread 10-50 µl of each transformation mix onto LB plates containing 50 µg/ml kanamycin and X-Gal (and IPTG, if using TOP10F´ cells). Be sure to plate two different volumes to ensure that at least one plate has well-spaced colonies. For plating small volumes, add 20 µl of S.O.C. medium to allow even spreading.
5. Incubate overnight at 37°C.

Analysis of Results

Hundreds of colonies from the vector + PCR insert reaction should be produced. Greater than ninety-five percent of these colonies will be white and 90% (or more) of these will contain the 750 bp insert when analyzed by EcoR I digestion and agarose gel electrophoresis.

Relatively few colonies will be produced in the vector-only reaction and most of these will be dark blue. You may observe a few white colonies. This results from removal of the 3´ deoxythymidine overhangs creating a blunt-end vector. Ligation (re-joining) of the blunt ends will result in disruption of the *lacZ*α reading frame leading to the production of white colonies.

Transformation Control

pUC19 plasmid is included to check the transformation efficiency of the One Shot® competent cells. Transform with 10 pg per 50 µl of cells using the protocols on pages 6-10.

Use LB plates containing 100 µg/ml ampicillin. Just before plating the transformation mix for electrocompetent cells, dilute 10 µl of the mix with 90 µl of S.O.C. medium.

Type of Cells	Volume to Plate	Transformation Efficiency
Chemically Competent	10 µl + 20 µl S.O.C.	~1 x 10^9 cfu/µg DNA
Electrocompetent	20 µl (1:10 dilution)	> 1 x 10^9 cfu/µg DNA

continued on next page

Factors Affecting Cloning Efficiency

Note that lower cloning efficiencies will result from the following variables. Most of these are easily correctable, but if you are cloning large inserts, you may not obtain the expected 95% (or more) cloning efficiency.

Variable	Solution
pH>9	Check the pH of the PCR amplification reaction and adjust with 1 M Tris-HCl, pH 8.
Incomplete extension during PCR	Be sure to include a final extension step of 7 to 30 minutes during PCR. Longer PCR products will need a longer extension time.
Cloning large inserts (>1 kb)	Try one or all of the following: Increase amount of insert. Incubate the TOPO® Cloning reaction longer. Gel-purify the insert (see page 20).
Excess (or overly dilute) PCR product	Reduce (or concentrate) the amount of PCR product.
Cloning blunt-ended fragments	Add 3´ A-overhangs to your blunt PCR product by incubating with *Taq* polymerase (page 22).
	Use the Zero Blunt® PCR Cloning Kit to clone blunt PCR products (Catalog no. K2700-20).
PCR cloning artifacts ("false positives")	TOPO® Cloning is very efficient for small fragments (< 100 bp) present in certain PCR reactions. Gel-purify your PCR product (page 20).
PCR product does not contain sufficient 3´ A-overhangs even though you used *Taq* polymerase	Increase the final extension time to ensure all 3´ ends are adenylated. *Taq* polymerase is less efficient at adding a nontemplate 3´ A next to another A. *Taq* is most efficient at adding a nontemplate 3´ A next to a C. You may have to redesign your primers so that they contain a 5´ G instead of a 5´ T (Brownstein *et al.*, 1996).

Appendix

Purifying PCR Products

Introduction

Smearing, multiple banding, primer-dimer artifacts, or large PCR products (>1 kb) may necessitate gel purification. If you intend to purify your PCR product, be extremely careful to remove all sources of nuclease contamination. There are many protocols to isolate DNA fragments or remove oligonucleotides. Refer to Current Protocols in Molecular Biology, Unit 2.6 (Ausubel et al., 1994) for the most common protocols. Two simple protocols are provided below for your convenience.

Using the S.N.A.P.™ MiniPrep Kit

The S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) allows you to rapidly purify PCR products from regular agarose gels. You will need to prepare 6 M sodium iodide, 10 mM sodium sulfite solution in sterile water before starting. Sodium sulfite prevents oxidation of NaI.

1. Electrophorese amplification reaction on a 1 to 5% regular TAE agarose gel.
   Note: Do not use TBE. Borate interferes with the NaI step (Step 2).
2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of 6 M NaI.
3. Add 1.5 volumes Binding Buffer (provided in the S.N.A.P.™ MiniPrep Kit).
4. Load solution (no more than 1 ml at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at full speed in a microcentrifuge and discard the supernatant.
5. If you have solution remaining from Step 3, repeat Step 4.
6. Add 900 µl of the Final Wash Buffer (provided in the S.N.A.P.™ MiniPrep Kit).
7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the supernatant. Repeat.
8. Elute the purified PCR product in 40 µl of TE or sterile water. Use 4 µl for the TOPO® Cloning reaction and proceed as described on page 5.

Quick S.N.A.P.™ Method

An easier method is to simply cut out the gel slice containing your PCR product, place it on top of the S.N.A.P.™ column bed, and centrifuge at full speed for 10 seconds. Use 1-2 µl of the flow-through in the TOPO® Cloning reaction (page 5). Be sure to make the gel slice as small as possible for best results.

continued on next page

Purifying PCR Products, continued

Low-Melt Agarose Method

Note that gel purification will result in a dilution of your PCR product. Use only chemically competent cells for transformation.

1. Electrophorese all of your PCR reaction on a low-melt TAE agarose gel (0.8 to 1.2%).
2. Visualize the band of interest and excise the band.
3. Place the gel slice in a microcentrifuge tube and incubate the tube at 65°C until the gel slice melts.
4. Place the tube at 37°C to keep the agarose melted.
5. Use 4 µl of the melted agarose containing your PCR product in the TOPO® Cloning reaction (page 5).
6. Incubate the TOPO® Cloning reaction **at 37°C for 5 to 10 minutes.** This is to keep the agarose melted.
7. Transform 2 to 4 µl directly into competent One Shot® cells using one of the methods described on pages 10-11.

Note

Note that the cloning efficiency may decrease with purification of the PCR product. You may wish to optimize your PCR to produce a single band.

Addition of 3´ A-Overhangs Post-Amplification

Introduction

Direct cloning of DNA amplified by proofreading polymerases into TOPO TA Cloning® vectors is often difficult because proofreading polymerases remove the 3´ A-overhangs necessary for TA Cloning®. Invitrogen has developed a simple method to clone these blunt-ended fragments.

Before Starting

You will need the following items:

- *Taq* polymerase
- A heat block equilibrated to 72°C
- Phenol-chloroform (optional)
- 3 M sodium acetate (optional)
- 100% ethanol (optional)
- 80% ethanol (optional)
- TE buffer (optional)

Procedure

This is just one method for adding 3´ adenines. Other protocols may be suitable.

1. After amplification with a proofreading polymerase, place vials on ice and add 0.7-1 unit of *Taq* polymerase per tube. Mix well. It is not necessary to change the buffer. A sufficient number of PCR products will retain the 3´ A-overhangs.
2. Incubate at 72°C for 8-10 minutes (do not cycle).
3. Place on ice and use immediately in the TOPO® Cloning reaction.

Note: If you plan to store your sample overnight before proceeding with TOPO® Cloning, extract your sample with an equal volume of phenol-chloroform to remove the polymerases. Ethanol-precipitate the DNA and resuspend in TE buffer using the starting volume of the PCR.

Note

You may also gel-purify your PCR product after amplification with a proofreading polymerase. After purification, add Taq polymerase buffer, dATP, and 0.5 unit of *Taq* polymerase. Incubate the reaction for 10-15 minutes at 72°C and use in the TOPO® Cloning reaction.

Recipes

LB (Luria-Bertani) Medium and Plates

Composition:

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55°C and add antibiotic if needed (50 μg/ml of either ampicillin or kanamycin).
4. Store at room temperature or at +4°C.

LB agar plates

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes at 15 psi.
3. After autoclaving, cool to ~55°C, add antibiotic (50 μg/ml of either ampicillin or kanamycin), and pour into 10 cm plates.
4. Let harden, then invert and store at +4°C in the dark.

Technical Service

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Product Qualification

Restriction Digest

Supercoiled pCR®2.1-TOPO® and pCR®II-TOPO® are qualified by restriction digest. The table below lists the restriction enzymes and the expected fragments.

Restriction Enzyme	pCR®2.1-TOPO®	pCR®II-TOPO®
*Hin*d III (linearizes)	3890 bp	3932 bp
Xba I (linearizes)	3890 bp	3932 bp
Nsi I	3890 bp	96, 3836 bp
Pst I	1167, 2723 bp	1167, 2765 bp
*Eco*R I and *Afl* III	408, 693, 2789bp	450, 693, 2789 bp

TOPO® Cloning Efficiency

Once the vectors have been adapted with topoisomerase I, they are lot-qualified using the control reagents included in the kit. Under conditions described on pages 17-18, a 750 bp control PCR product was TOPO®-Cloned into each vector and subsequently transformed into the One Shot® competent *E. coli* included with the kit.

Each lot of vector should yield greater than 95% cloning efficiency.

Primers

Both primers have been lot-qualified by DNA sequencing experiments using the dideoxy chain termination technique.

One Shot® Competent *E. coli*

All competent cells are qualified as follows:

- Cells are tested for transformation efficiency using the control plasmid included in the kit. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be ~1 x 10^9 cfu/µg DNA for chemically competent cells and >1 x 10^9 for electrocompetent cells.
- To verify the absence of phage contamination, 0.5-1 ml of competent cells are added to LB top agar and poured onto LB plates. After overnight incubation, no plaques should be detected.
- Untransformed cells are plated on LB plates 100 µg/ml ampicillin, 25 µg/ml streptomycin, 50 µg/ml kanamycin, or 15 µg/ml chloramphenicol to verify the absence of antibiotic-resistant contamination.

Purchaser Notification

Information for European Customers

The Mach1™-T1^R *E. coli* strain is genetically modified to carry the *lac*ZΔM15 *hsd*R *lac*X74 *rec*A *end*A *ton*A genotype. As a condition of sale, this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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Appendix H: Incubation Tube and Plate ID Log

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
1	1	1	W	1-Dec-05	1	
2	1	2	W	1-Dec-05	1	
3	1	3	W	1-Dec-05	1	
4	1	4	W	1-Dec-05	1	
5	1	5	W	1-Dec-05	1	
6	1	6	W	1-Dec-05	1	
7	1	7	W	1-Dec-05	1	
8	1	8	W	1-Dec-05	1	
9	1	9	W	1-Dec-05	1	
10	1	10	W	1-Dec-05	1	
11	1	11	B	1-Dec-05	1	
12	1	12	B	1-Dec-05	1	
13	1	13	B	1-Dec-05	1	
14	2	1	W	1-Dec-05	1	
15	2	2	W	1-Dec-05	1	
16	2	3	W	1-Dec-05	1	
17	2	4	W	1-Dec-05	1	
18	2	5	W	1-Dec-05	1	
19	2	6	W	1-Dec-05	1	
20	2	7	W	1-Dec-05	1	
21	2	8	W	1-Dec-05	1	
22	2	9	W	1-Dec-05	1	
23	2	10	W	1-Dec-05	1	
24	2	11	B	1-Dec-05	1	
25	2	12	B	1-Dec-05	1	
26	2	13	B	1-Dec-05	1	
27	3	1	W	1-Dec-05	1	
28	3	2	W	1-Dec-05	1	
29	3	3	W	1-Dec-05	1	
30	3	4	W	1-Dec-05	1	
31	3	5	W	1-Dec-05	1	
32	3	6	W	1-Dec-05	1	
33	3	7	W	1-Dec-05	1	
34	3	8	W	1-Dec-05	1	
35	3	9	W	1-Dec-05	1	
36	3	10	W	1-Dec-05	1	
37	BLANK			1-Dec-05	0	
38	BLANK			1-Dec-05	0	
39	BLANK			1-Dec-05	0	Inserted stick
40	BLANK			1-Dec-05	0	Inserted stick
41	31	1	W	10-Dec-05	1	
42	31	2	W	10-Dec-05	1	
43	31	3	W	10-Dec-05	1	
44	31	4	W	10-Dec-05	1	
45	31	5	W	10-Dec-05	1	
46	32	1	W	10-Dec-05	1	
47	32	2	W	10-Dec-05	1	
48	32	3	W	10-Dec-05	1	
49	32	4	W	10-Dec-05	1	
50	32	5	W	10-Dec-05	1	
51	33	1	W	10-Dec-05	1	
52	33	2	W	10-Dec-05	1	
53	33	3	W	10-Dec-05	1	
54	33	4	W	10-Dec-05	1	

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
55	33	5	W	10-Dec-05	1	
56	34	1	W	10-Dec-05	1	
57	34	2	W	10-Dec-05	1	
58	34	3	W	10-Dec-05	1	
59	34	4	W	10-Dec-05	1	
60	34	5	W	10-Dec-05	1	
61	35	1	W	10-Dec-05	1	
62	35	2	W	10-Dec-05	1	
63	35	3	W	10-Dec-05	1	
64	35	4	W	10-Dec-05	1	
65	35	5	W	10-Dec-05	1	
66	36	1	W	10-Dec-05	1	
67	36	2	W	10-Dec-05	1	
68	36	3	W	10-Dec-05	1	
69	36	4	W	10-Dec-05	1	
70	36	5	W	10-Dec-05	1	
71	37	1	W	10-Dec-05	1	
72	37	2	W	10-Dec-05	1	
73	37	3	W	10-Dec-05	1	
74	37	4	W	10-Dec-05	1	
75	37	5	W	10-Dec-05	1	
76	38	1	W	10-Dec-05	1	
77	38	2	W	10-Dec-05	1	
78	38	3	W	10-Dec-05	1	
79	38	4	W	10-Dec-05	1	
80	38	5	W	10-Dec-05	1	
81	39	1	W	10-Dec-05	1	
82	39	2	W	10-Dec-05	1	
83	39	3	W	10-Dec-05	1	
84	39	4	W	10-Dec-05	1	
85	39	5	W	10-Dec-05	1	
86	40	1	W	10-Dec-05	1	
87	40	2	W	10-Dec-05	1	
88	40	3	W	10-Dec-05	1	
89	40	4	W	10-Dec-05	1	
90	40	5	W	10-Dec-05	1	
91	41	1	W	10-Dec-05	1	
92	41	2	W	10-Dec-05	1	
93	41	3	W	10-Dec-05	1	
94	41	4	W	10-Dec-05	1	
95	41	5	W	10-Dec-05	1	
96	42	1	W	10-Dec-05	1	
97	42	2	W	10-Dec-05	1	
98	42	3	W	10-Dec-05	1	
99	42	4	W	10-Dec-05	1	
100	42	5	W	10-Dec-05	1	
101	43	1	W	10-Dec-05	1	
102	43	2	W	10-Dec-05	1	
103	43	3	W	10-Dec-05	1	
104	43	4	W	10-Dec-05	1	
105	43	5	W	10-Dec-05	1	
106	44	1	W	10-Dec-05	1	
107	44	2	W	10-Dec-05	1	

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
108	44	3	W	10-Dec-05	1	
109	44	4	W	10-Dec-05	1	
110	44	5	W	10-Dec-05	1	
111	45	1	W	10-Dec-05	1	
112	45	2	W	10-Dec-05	1	
113	45	3	W	10-Dec-05	1	
114	45	4	W	10-Dec-05	1	
115	45	5	W	10-Dec-05	1	
116	46	1	W	10-Dec-05	1	
117	46	2	W	10-Dec-05	1	
118	46	3	W	10-Dec-05	1	
119	46	4	W	10-Dec-05	1	
120	46	5	W	10-Dec-05	1	
121	47	1	W	10-Dec-05	1	
122	47	2	W	10-Dec-05	1	
123	47	3	W	10-Dec-05	1	
124	47	4	W	10-Dec-05	1	
125	47	5	W	10-Dec-05	1	
126	48	1	W	10-Dec-05	1	
127	48	2	W	10-Dec-05	1	
128	48	3	W	10-Dec-05	1	
129	48	4	W	10-Dec-05	1	
130	48	5	W	10-Dec-05	1	
131	49	1	W	10-Dec-05	1	
132	49	2	W	10-Dec-05	1	
133	49	3	W	10-Dec-05	1	
134	49	4	W	10-Dec-05	1	
135	49	5	W	10-Dec-05	1	
136	50	1	W	10-Dec-05	1	
137	50	2	W	10-Dec-05	1	
138	50	3	W	10-Dec-05	1	
139	50	4	W	10-Dec-05	1	
140	50	5	W	10-Dec-05	1	
141	51	1	W	10-Dec-05	1	
142	51	2	W	10-Dec-05	1	
143	51	3	W	10-Dec-05	1	
144	51	4	W	10-Dec-05	1	
145	51	5	W	10-Dec-05	1	
146	52	1	W	10-Dec-05	1	
147	52	2	W	10-Dec-05	1	
148	52	3	W	10-Dec-05	1	
149	52	4	W	10-Dec-05	1	
150	52	5	W	10-Dec-05	1	
151	53	1	W	10-Dec-05	1	
152	53	2	W	10-Dec-05	1	
153	53	3	W	10-Dec-05	1	
154	53	4	W	10-Dec-05	1	
155	53	5	W	10-Dec-05	1	
156	54	1	W	10-Dec-05	1	
157	54	2	W	10-Dec-05	1	
158	54	3	W	10-Dec-05	1	
159	54	4	W	10-Dec-05	1	
160	54	5	W	10-Dec-05	1	

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
161	55	1	BLNK	10-Dec-05	0	
162	55	2	BLNK	10-Dec-05	0	
163	55	3	BLNK	10-Dec-05	0	
164	55	4	BLNK	10-Dec-05	0	
165	55	5	BLNK	10-Dec-05	0	
166	56	1	BLNK	10-Dec-05	0	
167	56	2	BLNK	10-Dec-05	0	
168	56	3	BLNK	10-Dec-05	0	
169	56	4	BLNK	10-Dec-05	0	
170	56	5	BLNK	10-Dec-05	0	
171			BLNK	10-Dec-05	0	Empty tube
172			BLNK	10-Dec-05	0	Empty tube
173			BLNK	10-Dec-05	0	Empty tube
174			BLNK	10-Dec-05	0	Empty tube
175			BLNK	11-Dec-05	1	Empty tube
171	61	1	W	25-Dec-05	1	
172	61	2	W	25-Dec-05	1	
173	61	3	W	25-Dec-05	1	
174	61	4	W	25-Dec-05	1	
175	61	5	W	25-Dec-05	1	
176	63	1	W	25-Dec-05	1	
177	63	2	W	25-Dec-05	1	
178	63	3	W	25-Dec-05	1	
179	63	4	W	25-Dec-05	1	
180	63	5	W	25-Dec-05	1	
181	64	1	W	25-Dec-05	1	
182	64	2	W	25-Dec-05	1	
183	64	3	W	25-Dec-05	1	
184	64	4	W	25-Dec-05	1	
185	64	5	W	25-Dec-05	1	
186	65	1	W	25-Dec-05	1	
187	65	2	W	25-Dec-05	1	
188	65	3	W	25-Dec-05	1	
189	65	4	W	25-Dec-05	1	
190	65	5	W	25-Dec-05	1	
191	66	1	W	25-Dec-05	1	
192	66	2	W	25-Dec-05	1	
193	66	3	W	25-Dec-05	1	
194	66	4	W	25-Dec-05	1	
195	66	5	W	25-Dec-05	1	
196	67	1	W	25-Dec-05	1	
197	67	2	W	25-Dec-05	1	
198	67	3	W	25-Dec-05	1	
199	67	4	W	25-Dec-05	1	
200	67	5	W	25-Dec-05	1	
201	68	1	W	25-Dec-05	1	
202	68	2	W	25-Dec-05	1	
203	68	3	W	25-Dec-05	1	
204	68	4	W	25-Dec-05	1	
205	68	5	W	25-Dec-05	1	
206	69	1	W	25-Dec-05	1	
207	69	2	W	25-Dec-05	1	

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
208	69	3	W	25-Dec-05	1	
209	69	4	W	25-Dec-05	1	
210	69	5	W	25-Dec-05	1	
211	70	1	W	25-Dec-05	1	
212	70	2	W	25-Dec-05	1	
213	70	3	W	25-Dec-05	1	
214	70	4	W	25-Dec-05	1	
215	70	5	W	25-Dec-05	1	
216	71	1	W	25-Dec-05	1	
217	71	2	W	25-Dec-05	1	
218	71	3	W	25-Dec-05	1	
219	71	4	W	25-Dec-05	1	
220	71	5	W	25-Dec-05	1	
221	72	1	W	25-Dec-05	1	
222	72	2	W	25-Dec-05	1	
223	72	3	W	25-Dec-05	1	
224	72	4	W	25-Dec-05	1	
225	72	5	W	25-Dec-05	1	
226	73	1	W	25-Dec-05	1	
227	73	2	W	25-Dec-05	1	
228	73	3	W	25-Dec-05	1	
229	73	4	W	25-Dec-05	1	
230	73	5	W	25-Dec-05	1	
231	80	1	BLNK	25-Dec-05	0	
232	80	2	BLNK	25-Dec-05	0	
233	67	6	B	25-Dec-05		
234	63	6	B	25-Dec-05		
235	73	6	W	25-Dec-05	1	Joshua
236	73	7	W	25-Dec-05	1	Caleb
237	57	1	W	9-Jan-06	1	
238	57	2	W	9-Jan-06	1	
239	57	3	W	9-Jan-06	1	
240	57	4	W	9-Jan-06	1	
241	57	5	W	9-Jan-06	1	
242	58	1	W	9-Jan-06	1	
243	58	2	W	9-Jan-06	1	
244	58	3	W	9-Jan-06	1	
245	58	4	W	9-Jan-06	1	
246	58	5	W	9-Jan-06	1	
247	59	1	W	9-Jan-06	1	
248	59	2	W	9-Jan-06	1	
249	59	3	W	9-Jan-06	1	
250	59	4	W	9-Jan-06	1	
251	59	5	W	9-Jan-06	1	
252	60	1	W	9-Jan-06	1	
253	60	2	W	9-Jan-06	1	
254	60	3	W	9-Jan-06	1	
255	60	4	W	9-Jan-06	1	
256	60	5	W	9-Jan-06	1	
257	81	1	W	9-Jan-06	1	
258	81	2	W	9-Jan-06	1	
259	81	3	W	9-Jan-06	1	

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
260	81	4	W	9-Jan-06	1	
261	81	5	W	9-Jan-06	1	
262	82	1	W	9-Jan-06	1	
263	82	2	W	9-Jan-06	1	
264	82	3	W	9-Jan-06	1	
265	82	4	W	9-Jan-06	1	
266	82	5	W	9-Jan-06	1	
267	83	1	W	9-Jan-06	1	
268	83	2	W	9-Jan-06	1	
269	83	3	W	9-Jan-06	1	
270	83	4	W	9-Jan-06	1	
271	83	5	W	9-Jan-06	1	
272	84	1	W	9-Jan-06	1	
273	84	2	W	9-Jan-06	1	2ml of LB
274	84	3	W	9-Jan-06	1	2ml of LB
275	84	4	W	9-Jan-06	1	2ml of LB
276	84	5	W	9-Jan-06	1	2ml of LB
277	85	1	W	9-Jan-06	1	2ml of LB
278	85	2	W	9-Jan-06	1	2ml of LB
279	85	3	W	9-Jan-06	1	2ml of LB
280	85	4	W	9-Jan-06	1	2ml of LB
281	85	5	W	9-Jan-06	1	2ml of LB
282	86	1	W	9-Jan-06	1	2ml of LB
283	86	2	W	9-Jan-06	1	2ml of LB
284	86	3	W	9-Jan-06	1	2ml of LB
285	86	4	W	9-Jan-06	1	2ml of LB
286	86	5	W	9-Jan-06	1	2ml of LB
287	87	1	W	9-Jan-06	1	2ml of LB
288	87	2	W	9-Jan-06	1	2ml of LB
289	87	3	W	9-Jan-06	1	2ml of LB
290	87	4	W	9-Jan-06	1	2ml of LB
291	87	5	W	9-Jan-06	1	2ml of LB
292	88	1	W	9-Jan-06	1	2ml of LB
293	88	2	W	9-Jan-06	1	2ml of LB
294	88	3	W	9-Jan-06	1	2ml of LB
295	88	4	W	9-Jan-06	1	2ml of LB
296	88	5	W	9-Jan-06	1	2ml of LB
297	89	1	W	9-Jan-06	1	2ml of LB
298	89	2	W	9-Jan-06	1	2ml of LB
299	89	3	W	9-Jan-06	1	2ml of LB
300	89	4	W	9-Jan-06	1	2ml of LB
301	89	5	W	9-Jan-06	1	2ml of LB
302	90	1	W	9-Jan-06	1	2ml of LB
303	90	2	W	9-Jan-06	1	2ml of LB
304	90	3	W	9-Jan-06	1	2ml of LB
305	90	4	W	9-Jan-06	1	2ml of LB
306	90	5	W	9-Jan-06	1	2ml of LB
307	91	1	W	9-Jan-06	1	2ml of LB
308	91	2	W	9-Jan-06	1	2ml of LB
309	91	3	W	9-Jan-06	1	2ml of LB
310	91	4	W	9-Jan-06	1	2ml of LB
311	91	5	W	9-Jan-06	1	2ml of LB
312	92	1	W	9-Jan-06	1	2ml of LB

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
313	92	2	W	9-Jan-06	1	2ml of LB
314	92	3	W	9-Jan-06	1	2ml of LB
315	92	4	W	9-Jan-06	1	2ml of LB
316	92	5	W	9-Jan-06	1	2ml of LB
317	93	1	W	9-Jan-06	1	2ml of LB
318	93	2	W	9-Jan-06	1	2ml of LB
319	93	3	W	9-Jan-06	1	2ml of LB
320	93	4	W	9-Jan-06	1	2ml of LB
321	93	5	W	9-Jan-06	1	2ml of LB
322	94	1	W	9-Jan-06	1	2ml of LB
323	94	2	W	9-Jan-06	1	2ml of LB
324	94	3	W	9-Jan-06	1	2ml of LB
325	94	4	W	9-Jan-06	1	2ml of LB
326	94	5	W	9-Jan-06	1	2ml of LB
327	95	1	W	9-Jan-06	1	2ml of LB
328	95	2	W	9-Jan-06	1	2ml of LB
329	95	3	W	9-Jan-06	1	2ml of LB
330	95	4	W	9-Jan-06	1	2ml of LB
331	95	5	W	9-Jan-06	1	2ml of LB
332	96	1	W	9-Jan-06	1	2ml of LB
333	96	2	W	9-Jan-06	1	2ml of LB
334	96	3	W	9-Jan-06	1	2ml of LB
335	96	4	W	9-Jan-06	1	2ml of LB
336	96	5	W	9-Jan-06	1	2ml of LB
337	97	1	W	9-Jan-06	1	2ml of LB
338	97	2	W	9-Jan-06	1	2ml of LB
339	97	3	W	9-Jan-06	1	2ml of LB
340	97	4	W	9-Jan-06	1	2ml of LB
341	97	5	W	9-Jan-06	1	2ml of LB
342	98	1	W	9-Jan-06	1	2ml of LB
343	98	2	W	10-Jan-06	1	
344	98	3	W	10-Jan-06	1	
345	98	4	W	10-Jan-06	1	
346	98	5	W	10-Jan-06	1	
347	99	1	W	10-Jan-06	1	
348	99	2	W	10-Jan-06	1	
349	99	3	W	10-Jan-06	1	
350	99	4	W	10-Jan-06	1	
351	99	5	W	10-Jan-06	1	
352	100	1	W	10-Jan-06	1	
353	100	2	W	10-Jan-06	1	
354	100	3	W	10-Jan-06	1	
355	100	4	W	10-Jan-06	1	
356	100	5	W	10-Jan-06	1	
357	101	1	BLNK	10-Jan-06	0	
358	101	2	BLNK	10-Jan-06	0	
359	BLNK	1	BLNK	10-Jan-06	0	Place loop in LB solutio
360	BLNK	2	BLNK	10-Jan-06	0	
361	102	1	W	12-Jan-06	1	
362	102	2	W	12-Jan-06	1	
363	102	3	W	12-Jan-06	1	
364	102	4	W	12-Jan-06	1	

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
365	102	5	W	12-Jan-06	1	
366	103	1	W	12-Jan-06	1	
367	103	2	W	12-Jan-06	1	
368	103	3	W	12-Jan-06	1	
369	103	4	W	12-Jan-06	1	
370	103	5	W	12-Jan-06	1	
371	104	1	W	12-Jan-06	1	
372	104	2	W	12-Jan-06	1	
373	104	3	W	12-Jan-06	1	
374	104	4	W	12-Jan-06	1	
375	104	5	W	12-Jan-06	1	
376	105	1	W	12-Jan-06	1	
377	105	2	W	12-Jan-06	1	
378	105	3	W	12-Jan-06	1	
379	105	4	W	12-Jan-06	1	
380	105	5	W	12-Jan-06	1	
381	106	1	W	12-Jan-06	1	
382	106	2	W	12-Jan-06	1	
383	106	3	W	12-Jan-06	1	
384	106	4	W	12-Jan-06	1	
385	106	5	W	12-Jan-06	1	
386	107	1	W	12-Jan-06	1	
387	107	2	W	12-Jan-06	1	
388	107	3	W	12-Jan-06	1	
389	107	4	W	12-Jan-06	1	
390	107	5	W	12-Jan-06	1	
391	108	1	W	12-Jan-06	1	
392	108	2	W	12-Jan-06	1	
393	108	3	W	12-Jan-06	1	
394	108	4	W	12-Jan-06	1	
395	108	5	W	12-Jan-06	1	
396	109	1	W	12-Jan-06	1	
397	109	2	W	12-Jan-06	1	
398	109	3	W	12-Jan-06	1	
399	109	4	W	12-Jan-06	1	
400	109	5	W	12-Jan-06	1	
401	110	1	W	12-Jan-06	1	
402	110	2	W	12-Jan-06	1	
403	110	3	W	12-Jan-06	1	
404	110	4	W	12-Jan-06	1	
405	110	5	W	12-Jan-06	1	
406	111	1	W	12-Jan-06	1	
407	111	2	W	12-Jan-06	1	
408	111	3	W	12-Jan-06	1	
409	111	4	W	12-Jan-06	1	
410	111	5	W	12-Jan-06	1	
411	112	1	W	12-Jan-06	1	
412	112	2	W	12-Jan-06	1	
413	112	3	W	12-Jan-06	1	
414	112	4	W	12-Jan-06	1	
415	112	5	W	12-Jan-06	1	
416	113	1	W	12-Jan-06	1	
417	113	2	W	12-Jan-06	1	

Incubation Tube ID	Plate ID	Coloney	Coloney Color	Date	Tube Cloudy	Notes
418	113	3	W	12-Jan-06	1	
419	113	4	W	12-Jan-06	1	
420	113	5	W	12-Jan-06	1	
421	114	1	W	12-Jan-06	1	
422	114	2	W	12-Jan-06	1	
423	114	3	W	12-Jan-06	1	
424	114	4	W	12-Jan-06	1	
425	114	5	W	12-Jan-06	1	
426	115	1	W	12-Jan-06	1	
427	115	2	W	12-Jan-06	1	
428	115	3	W	12-Jan-06	1	
429	115	4	W	12-Jan-06	1	
430	115	5	W	12-Jan-06	1	
431	116	1	W	12-Jan-06	1	
432	116	2	W	12-Jan-06	1	
433	116	3	W	12-Jan-06	1	
434	116	4	W	12-Jan-06	1	
435	116	5	W	12-Jan-06	1	
436	117	1	BLNK	12-Jan-06	0	
437	117	2	BLNK	12-Jan-06	0	
438	118	1	BLNK	12-Jan-06	0	
439	118	2	BLNK	12-Jan-06	0	
440	BLNK	BLNK	BLNK	12-Jan-06	0	
441	BLNK	BLNK	BLNK	12-Jan-06	0	

Appendix I: Promega Restriction Enzyme Specification Sheet

Promega

Usage Information

Introduction

Restriction enzymes, also referred to as restriction endonucleases, are enzymes which recognize short, specific (often palindromic) DNA sequences. They cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to their recognition sequences. Most restriction enzymes (REs) will not cut DNA that is **methylated** on one or both strands of their **recognition site**, although some require substrate methylation.

Each restriction enzyme has specific requirements to achieve optimal activity. Ideal storage and assay conditions favor the most activity and highest fidelity in a particular enzyme's function. Conditions such as temperature, pH, enzyme cofactor(s), salt composition and ionic strength affect enzyme activity and stability. Two buffers usually accompany each of Promega's restriction enzymes. One buffer is the optimal reaction buffer which may be from the **4-CORE® System** (Reaction Buffers A, B, C, D) or one of the other optimal buffers (Reaction Buffers E–L), and the other is the MULTI-CORE™ Buffer. The supplied optimal buffer always yields 100% activity for the enzyme it accompanies, and serves as the specific reaction buffer for individual digests with that enzyme. **The MULTI-CORE™ Buffer**, which is designed for broad compatibility with many REs, is provided with enzymes that have 25% or greater activity in this buffer. The MULTI-CORE™ Buffer is useful for multiple digests because it generally yields more activity for more enzyme combinations than any of the other buffers, but sometimes with a compromise in activity. Multiple digests using REs with significantly different buffer requirements may require a sequential reaction with the addition of RE buffer or salt before the second enzyme is used.

DNA Substrate Considerations

DNA substrates commonly used for restriction enzyme digestion include DNA from bacteriophage lambda, bacterial plasmid DNA and genomic DNA. Lambda DNA is a linear DNA form that is an industry standard for measuring and expressing unit activity for many restriction enzymes. Compared to linear DNA, intact supercoiled plasmid DNA (and DNAs with a large number of the target restriction site) require more units of enzyme (two- to tenfold) per microgram than the DNA used in the enzyme's activity assay.

PCR products and oligonucleotides are relatively small compared with DNA used for defining RE units. Therefore, when using these substrates in a restriction digest, it is essential to take into consideration the molar concentration of enzyme recognition sites and not just the mass of DNA. Also, some REs require flanking bases surrounding the core RE recognition site. This is problematic when it is necessary to cut an oligonucleotide or a fragment of DNA with an RE site near its end. When PCR cloning strategies include the use of primers containing an RE site, care is necessary in designing the primer with adequate DNA surrounding the core RE recognition sequence.

In addition to the form and original source of the DNA, the purity is another factor that must be considered. Depending on the purification method and the handling of the DNA, it may contain varying amounts of contaminants that affect restriction enzyme digestion and analysis. Contaminants may include other types of DNA, nucleases, salts and inhibitors of restriction enzymes. The effect of a contaminant on an RE digest is generally dose-dependent: i.e., the inhibitory effects will increase with the volume of DNA added to the restriction enzyme reaction. Relatively pure DNA is required for efficient restriction enzyme digestion. Contaminating nucleases are usually activated only after the addition of salts (e.g., restriction enzyme buffer) to the DNA solution. Therefore, appropriate control reactions should always be run in parallel with the restriction digest. Buffer solutions containing EDTA in low concentrations (1mM) are often used to protect DNA from nuclease degradation during storage, but the EDTA can interfere with restriction enzyme digestion if the final concentration of EDTA in the reaction is too high. This situation usually results when the concentration of the substrate DNA is low and it is necessary to use a large volume of DNA in the digest. In such cases, it is best to concentrate the DNA (e.g., by ethanol precipitation). The organic solvents, salts, detergents and chelating agents that are sometimes used during the purification of DNA can also interfere with restriction enzyme activity if they carry over into the final DNA solution. Dialysis and/or ethanol precipitation with 2.5M ammonium acetate (final concentration before adding ethanol) followed by drying and resuspension can remove many of these substances. While relatively pure DNA is required for efficient restriction enzyme digestion, addition of acetylated BSA to a final concentration of 0.1mg/ml can sometimes improve the quality and efficiency of enzyme assays containing impure DNA and we recommend that it be included in all digests.

Enzyme Storage, Handling and Use

Maintain the sterility of reagents used in the RE digest as well as any tools (e.g., tubes, pipette tips) used with those reagents. Restriction enzymes should be stored in a non-frost-free freezer, except for a brief period during use, when they should be kept on ice. The restriction enzyme is usually the last reagent added to a reaction, to ensure that it is not exposed to extreme conditions. When many similar digests are being prepared, it may be convenient to create premixes of common reagents.

Before assembling the restriction digest, thoroughly mix each component to be added to the reaction and then centrifuge the tubes of reagents briefly to collect the contents in the bottom of the tube. The reaction components should also be mixed after addition of the enzyme to the digest. While high salt buffers and glycerol-containing reagents are difficult to mix, all solutions containing restriction enzymes must be mixed gently to avoid inactivating the enzyme.

Setting up a Restriction Enzyme Digest

An analytical scale restriction enzyme digest is usually performed in a volume of 20µl on 0.2–1.5µg of substrate DNA, using a two- to tenfold excess of enzyme over DNA. If an unusually large volume of DNA or enzyme is used, aberrant results may occur and may or may not be readily recognized. The following is an example of a typical RE digest. In a sterile tube, assemble in order:

sterile, deionized water	16.3µl
RE 10X Buffer	2µl
Acetylated BSA, 10µg/µl	0.2µl
DNA, 1µg/µl	1µl

Mix by pipetting, then add:

Restriction Enzyme, 10u/µl	0.5µl
final volume	20µl

Mix gently by pipetting, close the tube and centrifuge for a few seconds in a microcentrifuge. Incubate at the optimum temperature for 1–4 hours.

Add 4µl of 6X loading buffer and proceed to gel analysis. Note that overnight digests are usually unnecessary and may result in degradation of the DNA.

Experimental Controls

Experimental controls are necessary to identify, understand and explain problems or inconsistencies in results. The following controls are commonly used in parallel with RE digests: (i) uncut experimental DNA, (ii) digest of commercially supplied control DNA, (iii) no-enzyme "mock" digest, (iv) 1 or 2 different size markers in more than one lane per gel (i.e., different locations).

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Appendix J: Study Event Log

Season	Location	Section	DNA Template	PCR I	Gel I	PCR Pool	Plate Culture	Sample ID	Gel ID	DNA Conc. (NANO) ng/l	DNA Ident. (Blast)	% identity	E value
Winter	Valle Green	Top	61	232		262P	61	160		417.99	AF316785.1	97.23	0
								161		393.29	AY834311.1	96.86	0
								162		512.57	AY425772.1	95.83	0
								163		421.42	AY694491.1	93.14	0
								164		409.55			
							63	165		375.59			
								166		416.67	AJ890100.1	94.62	0
								167		444.24	AJ863206.1	95.72	6E-177
								168		374.55	AB179670.1	89.78	2E-157
								169		399.46	AY922126.1	99.36	0
								223		337.47			
				262			64	170		445.99	AY921485.1	89.2	5E-148
								171		270.15	DQ158100.1	96.27	0
								172		427.66	AF280847.1	84.2	2E-116
								173		343.48	AY921916.1	96.82	0
								174		405	DQ093934.1	96.73	0
		Middle	62	264		278P	65	175		374.01	DQ129016.1	96.99	2E-121
								176		484.4	AY921916.1	96.61	0
								177		486.16	AY922024.1	90.38	0
								178		434.82	DQ154621.1	98.51	0
								179		348.62	AY546507.1	87.02	2E-147
							66	180		436.9	AY989063.1	96.73	3E-155
								181		256.02	AY037562.1	98.54	0
								182		372.44	DQ138955.1	97.61	0
								183		371.92	AJ233911.1	98.1	0
								184		395.02	DQ154627.1	98.04	0
				278			67	185		392.27	AJ863233.1	94.32	0
								186		378.66	AY177763.1	97.58	0
								187		417.35	DQ154301.1	98.87	0
								188		370.95	DQ165096.1	94.46	0
								189		324.44	AY043904.1	96.97	0
								222		282.64	AJ784135.1	91.89	1E-17
		Lower	63	226		265P	68	190		345.18	AY592619.1	96	0
								191		383.31	AY584744.1	88.24	3E-154
								192		389.07	DQ223088.1	91.58	9E-180
								193		389.56			
								194		257.88	AB177255.1	90.08	3E-180
							69	195		348.19	AB177334.1	87.66	3E-174
								196		399.33	AY869683.1	90.15	0
								197		357.2	AY922084.1	89.19	0
								198		403.84			
								199		419.81	AY354168.1	84.8	6E-142
				265			70	200		331.5	AF407200.1	95.64	0
								201		387.85	AF422607.1	87.94	5E-153
								202		391.69	DQ066981.1	91.6	0
								203		375.03	AJ306790.1	91.06	0
								204		285.98	AY360666.1	96.05	0
	WPAFB (Treatmen	Top	58	259		279P	71	205		377.05	Y07580.2	98.65	0
								206		362.82			
								207		357.35	AY395438.1	94.32	0
								208		349.78	AF523332.1	98.23	0
								209		232.44	AY694610.1	89.34	0
							72	210		371.3	AY037562.1	98.96	0
								211		320.58	AY955095.1	98.27	0
								212		377.2	DQ067007.1	87.46	4E-173
								213		328.25	AY955095.1	99.2	0
								214		384.78	AB238029.1	96.46	0
				279			73	215		313.14	AF388341.1	90.68	0
								216		321.59	AY921821.1	97.29	0
								217		229.59	AY754024.1	96.8	0
								218		243.1	AY464548.1	90.86	0
								219		367.91	AY694604.1	96.13	0
								224		374.43	DQ154562.1	88.57	0
								225		272.7	AY135927.1	96.28	0
		Middle	59	260		280P	111	394		206.39	AB116121.1	97.46	0
								395		214.1	AB043854.1	95.68	0
								396		211.51	AY081988.1	98.92	0
								397		165.88	AY235435.1	90.82	0
								398		212.92	AJ863242.1	99.42	0
							112	399		214.09	DQ088792.1	96.38	0
								400		218.82	AJ318159.1	88.1	3E-30
								401		236.06	DQ154651.1	99.23	0
								402		267.27	AJ252662.1	99.43	0
								403		230	AJ863185.1	97.83	0
				280			113	404		194.18	AY607176.1	96.33	0
								405		204.74			
								406		201.09	DQ128951.1	79.18	4E-38
								407		200.95	AY570583.1	98.69	0
								408		188.83	DQ154377.1	98.82	0
		Lower	54	246		258P	31	25		199.06	AY221615.1	98.25	0
								26		274.96	AF388362.1	97.77	2E-126
								27		230.47	AJ853514.1	81.13	2E-97
								28		289.28	AM159457.1	98.13	0
								29		312.45	AB177205.1	96.03	2E-61
							32	30		249.84	DQ228372.1	97.13	0
								31		146.06	AF013558.2	99.77	0
								32		275.9	AY515486.1	92.58	0
								32		275.9	AB234247.1	94.26	0
								33					
								34		584.08	DQ093937.1	89.55	2E-176
				258			33	35		192.92	DQ125814.1	97.5	0
								36		252.04			
								37					
								38		200.24	AY037562.1	98.75	0
								39		228.77	AB234261.1	91.06	0

Season	Location	Section	DNA Template	PCR I	Gel I	PCR Pool	Plate Culture	Sample ID	Gel I	DNA Conc (NANO) ng/	DNA Ident. (Blast)	% identity	E value
Spring	Valle Gree	Top	64	235		266P	43	85					
								86		220.79	AB177205.1	93.33	1E-52
								87					
								88		212.89	AY221613.1	99.21	0
								89		243.22	AY947554.1	97.65	0
							44	90		235.19	AY959162.1	99.2	0
								91		171.78	AY464463.1	99.42	0
								92		246.08	AY921916.1	91.51	4E-178
				266				93		253.34	AJ582053.1	98.94	0
								94		243.56	AB089951.1	98.88	0
							45	95		173.77	DQ108394.1	98.5	0
								96		155.33	AJ518553.1	87.63	3E-54
								97		340.17	AY834304.1	97.88	0
								98					
								99		334.25	AF443586.1	95.09	0
		Middle	65	228		267P	46	100					
								101		231.8	DQ201592.1	92.86	0
								102		192.25	AY592366.1	92.71	0
								103		357.91	DQ165091.1	92.64	0
								104		253.44	DQ145139.1	94.15	0
							47	105		241.61	AY150879.1	96.39	0
								106		406	AB099988.1	84.69	1E-13
								106		406	AF320959.1	95.45	0
								107		329.32	AY922150.1	95.71	0
				267				108		180.31	AJ863184.1	91.55	1E-166
								109		238.06	AY307861.1	91.83	2E-176
							48	110		300.52	DQ110128.1	86.09	1E-129
								111		337.11	DQ128791.1	86.02	4E-148
								112		311.38	AY632433.1	95.65	1E-163
								113		334.18	AY508257.1	95.24	0
								114		471.8	DQ154422.1	99.2	0
		Lower	19	284		285P	49	115		395.22	DQ093903.1	97.57	0
								116		227.13	AY921949.1	97.95	0
								117		341.55	DQ154527.1	99.16	0
								118		333.5	DQ154634.1	99.16	0
								119		382.27			
							50	120		495.51	DQ018805.1	76.62	4E-29
								121		228.78	AY922159.1	98.5	0
								122		271.97	AJ252611.1	97.02	0
				285				123		363.62	AY921838.1	98.31	0
								124		458.39	AB240347.1	83.62	3E-125
							51	125		220.19	DQ154361.1	99.43	0
								126		255.24	AJ863236.1	95.93	0
								127		539.81	AJ875423.1	90.47	2E-162
								128		569.72	DQ129053.1	95.98	0
								129		363.84	AY493936.1	82.46	1E-58
	WPAFB (Treatmen	Top	38	253		254P	34	40		356.49	AJ853938.1	100	4E-44
								41		265.12	AY921881.1	92.41	0
								42		427.52	AJ232848.1	87.5	1E-93
								43		257.57	AY162061.1	99.61	0
								44		250.64	AY221611.1	99.6	0
							35	45		306.31	AY869683.1	84.34	3E-135
								46		393.63			
								47		360	AY186808.1	91.77	0
				254				48		407.97	AJ876729.1	85.49	1E-118
								49		324.12	DQ128365.1	90.15	2E-166
							36	50		178.78	AY221615.1	97.87	0
								51		144.96	AJ745078.1	92.31	2E-25
								52		335.07	AY694600.1	88.94	6E-157
								53					
								54		192.85	DQ128791.1	97.06	0
		Middle	40	255		256P	37	55		291.36	AJ863189.1	96.6	0
								56		285.77	AB201587.1	95.3	0
								57		298.27	AY568514.1	98.11	0
								58		263.66	AY186863.1	99.14	0
								59		237.63	DQ154451.1	99.42	0
							38	60		245.36	AJ252644.1	96.64	0
								61		262.73	AM159379.1	95.59	0
								62		310.7	DQ297986.1	98.62	0
				256				63		296.8	AY043899.1	97.4	0
								64		315.25	AY162061.1	96.35	0
							39	65		272.85	AJ544074.1	97.3	1E-43
								66		345.22	AY921940.1	98.48	0
								67		296.31	Z95708.1	98.14	0
								68		281.54	DQ125648.1	92.36	0
								69		228.65	AB240266.1	98.32	0
		Lower	33	251		252P	40	70		282.55	DQ191697.1	87.08	2E-162
								71		157.68	AB187506.1	87.35	7E-125
								72		223.3	AB240264.1	90.4	0
								73		288.72	AF317771.1	91.67	0
								74		289.64	AB177319.1	89.31	0
							41	75		304.29	AM167966.1	82.62	4E-133
								76		171.72	DQ083105.1	98.65	0
								77					
								78		228.24	AJ863216.1	99.54	0
				252				79		161.69	AF351238.1	93.46	0
							42	80					
								81		227.68	AB234248.1	98.07	0
								82		234.96	AJ544074.1	100	2E-40
								83		355.84	DQ154377.1	97.27	0
								84		243.21	AY834348.1	99.77	0

Season	Location	Section	DNA Templa	PCR I	Gel I	PCR Po	Plate Culture	Sample ID	Gel ID	DNA Conc. (NANO) ng/	DNA Ident. (Blast)	% identity	E value
								232					
							83	267		306.38	AB240474.1	88.38	5E-161
								268		241.97	AB240225.1	93.86	0
				273				269		362.94			
								270		277.5	AM159243.1	92.63	0
								233		180.13	DQ154525.1	91.68	0
							84	271		381.27	AY957930.1	96.05	0
		Top	70			360P		272		353.52	AF423245.1	83.78	1E-27
								273		426.6			
								274		337.38	AJ390466.1	92.62	4E-167
				360				234		475.59	DQ154420.1	95.57	3E-139
							85	275		413.8	AY989545.1	98.33	0
								276		391.65	DQ065070.1	83.49	2E-126
								277		381.23	AJ863236.1	89.29	5E-176
								278		383.81			
								379		257.22	AY568768.1	93.07	0
							108	380		230.68	AB187912.1	98.13	0
								381		272.29	DQ058675.1	98.87	0
				362				382		272.04	DQ191735.1	88.59	2E-166
								383		210.82	DQ154525.1	98.13	0
								384		249.52	DQ154525.1	96.66	0
	Valle Gree	Middle	71			370P	109	385		256.73	AJ506120.1	97.77	0
								386		222.21	AY921838.1	98.11	0
								387		254.55	AF292999.1	92.36	0
				370				388		186.67	AF392798.1	84.52	8E-125
								389		247.48	AY493917.1	97.5	0
							110	390		290.81	DQ154515.1	93.64	0
								391		172.8	DQ154581.1	92.45	0
								392		235.06	DQ154525.1	99.07	0
								393		319.67	AY921654.1	94.61	0
								409		267.58	AY037562.1	97.71	0
							114	410		287.53	AY214753.1	86.35	6E-121
								411		167.04	AJ009448.1	88.17	2E-170
				275				412		244.8	AF320959.1	94.41	0
								413		237.9	AY592619.1	96	0
								414		296.77	AM086107.1	96.4	0
							115	415		265.05	AY711533.1	88.48	6E-151
		Lower	72			283P		416		280.23	AM180059.1	91.97	0
								417		289.03	AM086107.1	96.93	0
								418		289.85	AF280847.1	86.71	1E-133
				283				419		285.2	DQ138957.1	96.45	0
							116	420		258.77	AF407200.1	95.62	0
								421		240.39	AY555810.1	93.32	0
								422		242.3			
								423		178.61	AF320959.1	93.83	0
								130		401.15	AY568857.1	94.85	0
							52	131		268.96	AB186806.1	89.33	0
								132		335.22	AY917425.1	89.8	0
				238				133		463.77	AY921867.1	94.52	0
								134		356.68	AY869683.1	83.11	1E-124
								135		320.61	AY150879.1	97.89	0
							53	136		454.61	AB177192.1	90.36	4E-168
		Top	67			270P		137		369.53	AY326608.1	98.26	0
								138		498.32	CR933146.1	87.25	7E-151
								139		184.11	AY725249.1	98.42	0
				270				140		245.61	AB201621.1	94.53	0
							54	141		286.04	DQ129631.1	96.79	0
								142		283.69	AY917287.1	93.99	0
								143		317.1	DQ125669.1	97.11	0
								144		235.23	AY921704.1	88.94	1E-63
								229					
							60	255		265.66			
								256		295.54	AB240358.1	95.58	0
				343				257		317.79	AY542229.1	89.89	0
								258		306.33	DQ128781.1	90.27	9E-164
								230					
							81	259		327.32	AY988887.1	90.58	0
		Middle	68			366P		260		266.28	AY387299.1	93.71	7E-145
								261		343.72	AY921904.1	88.48	9E-169
	WPAFB (Treatmen							262		227.67	AY917847.1	91.06	0
				366				231					
							82	263		283.48	AB087523.1	89.11	2E-76
								264		234.42	DQ154634.1	89.58	2E-175
								265		321.58	AJ006090.1	94.81	0
								266		334.87	AY043947.1	92.13	0
								1	1	292.79	DQ195646.1	91.53	3E-159
								2	1	277.1	DQ066989.1	96.71	0
								3	1	444.22			
							1	4	1	213.17	AY395377.1	96.84	0
								5		273.72	AJ585959.1	98.93	0
				240				6					
								16	0,0	313.1	AM159269.1	98.13	0
								17	0,0	277.2	AY177765.1	90.26	0
								7	1	347.51	AB240491.1	89.33	0
								8	1	415.48	AY953235.1	92.89	0
		Lower	69			272P		9	1	304.34	AY043899.1	95.2	0
								10	1	321.45	AB245336.1	95.66	0
							2	11		629.51			
								12		253.79	AY569777.1	99.12	4E-49
								18		384.56			
				272				19		263.2	AY150868.1	97.91	0
								20		374.85			
								21		210.02	AY221615.1	98.45	0
							3	22		395.66	AY221057.1	97.18	0
								23		260.03	AY494658.1	91.04	8E-66

Season	Location	Section	DNA Template	PCR I	Gel I	PCR Pool	Plate Culture	Sample ID	Gel I	DNA Conc (NANO) ng/	DNA Ident. (Blast)	% identity	E value
Fall	Valle Gree	Top	47	353		354P	95	315		475.43	DQ110117.1	93.46	0
								316		397.16	AB240347.1	83.71	9E-130
								317		387.75	AY710627.1	94.75	0
								318		423.29	AJ853599.1	92.91	2E-166
								319		410.8			
							96	320		409.1	AY869683.1	89.62	2E-180
								321		435.9	AF141536.1	97.8	0
								322		439.28	AB234280.1	93.9	0
								323		428.21	AY043899.1	97	0
				354				324		381.56	DQ093903.1	89.01	0
							97	325		384.77	AY921913.1	88.85	9E-170
								326		446.42	AY921916.1	96.41	0
								327		380.26	DQ154336.1	89.33	0
								328		372.47			
								329		369.08	AY568908.1	89.72	2E-175
		Middle	49	355		356P	98	330		392.99	AY493917.1	95.11	0
								331		239.49	AY921859.1	93.76	0
								332		344.13	DQ128372.1	86.93	2E-96
								333		326.06	AY150879.1	93.52	0
								334		268.08	AJ544074.1	98.17	1E-44
							99	335		255.75	AJ519644.1	97.16	0
								336		272.25	AY154623.1	82.49	2E-27
								337		259.43	AF422593.1	84.45	2E-66
				356				338		276.29	DQ067029.1	91.8	0
								339		236.6	DQ093926.1	96.7	0
							100	340		292.26	AB240510.1	90.25	2E-170
								341		176.12	AY221065.1	94.22	1E-177
								342		242.15	AJ551170.1	99.8	0
								343		173.29	AY917420.1	89.48	1E-177
								344		351.01	DQ154377.1	97.28	0
		Lower	114	316		317P	102	349		291.7	AJ005994.1	95.68	0
								350		307.51	AB179676.1	85.99	3E-110
								351		311.18	AB177205.1	91.77	6E-52
								352		275.41	AY354188.1	94.2	5E-177
								353		262.58	DQ154336.1	97.53	0
							103	354		297.13	DQ123789.1	88.87	1E-153
								355		316.7	AY214805.1	82.64	2E-67
								356		257.23	DQ093903.1	96.8	0
								357		239.27	AY921703.1	98.27	0
				317				358		307.72	AM167972.1	84.39	5E-117
							104	359		223.76	AB234266.1	85.45	7E-151
								360		314.95			
								361		269.11	AY283125.1	90.87	2E-166
								362		250.21			
								363		245.42	AB177205.1	98.32	3E-50
	WPAFB (Treatmer	Top	41	347		348P	86	235		397.65			
								279		400.11	AB185003.1	86.92	3E-113
								280		423.41	AJ544074.1	95.54	6E-41
								281		403.86	DQ093950.1	90.85	9E-169
								282		371.03	AY013611.1	88.66	1E-177
							87	236					
								283		402.1	AJ431217.1	90.51	1E-47
								284		418.64	AY921908.1	93.81	0
								285		428.92	AJ704365.1	89.3	2E-121
				348				286		382.73	DQ329344.1	95.81	0
							88	237		287.57	AJ888558.1	95.85	0
								287		419.06	DQ076455.1	96.93	0
								288		417.48	AF523321.1	91.28	2E-136
								289		428.9	DQ202161.1	90.52	0
								290		431.72	AY921569.1	95.5	0
		Middle	43	349		350P	89	238		253.66	DQ154551.1	88.69	6E-121
								291		454.59	AY144276.1	95.07	0
								292		350.84	AJ863208.1	81.02	4E-83
								293		430.75	DQ201599.1	91.58	0
								294		498.63	AF446261.1	91.79	0
							90	239					
								295		467.62	AY037562.1	97.89	0
								296		388.46	DQ129485.1	98.83	0
								297		453.09	DQ165096.1	94.25	0
				350				298		450.19	AJ292615.1	98.07	0
							91	240		306.66	AY607163.1	84.94	2E-140
								299		509.6	DQ154442.1	99.03	0
								300		400.92	AY568907.1	97.24	0
								301		390.21			
								302		339.23	AY456903.1	97.02	0
		Lower	46	351		352P	92	241		259.76	DQ211504.2	96.32	9E-174
								303		434.59	AJ567598.1	80	1E-78
								304		400.7	DQ154377.1	98.83	0
								305		355.5	DQ248291.1	92.06	0
								306		413.19			
							93	242		218.2	AJ617866.1	98.4	9E-154
								307		348.16	AY360666.1	95.37	0
								308		356.71	DQ128736.1	98.04	0
								309		348.96	DQ154377.1	99.12	0
				352				310		441.46	AM159379.1	93.38	0
							94	243		294.69	AY921830.1	90.37	0
								311		438.95	AY921769.1	97.68	0
								312		352.74	DQ335011.1	93.42	1E-162
								313		392.33	DQ154633.1	88.69	5E-162
								314		479.87	AJ863186.1	93.56	1E-133

Appendix K: Beckman Coulter GenomeLab™ Methods Development Kit

BECKMAN COULTER.

608019-AR
March 2005

GenomeLab™ Methods Development Kit
Dye Terminator Cycle Sequencing

The GenomeLab Methods Development Kit (MDK) offers multiple sequencing chemistries for performing DNA sequencing. **It consists of a set of core reagents plus two dNTP solutions: dNTP(I) Mix containing dITP and dNTP(G) Mix containing dGTP.** Separate cycling conditions are also used for these two different chemistries. The dITP chemistry offers the full capabilities of the previous CEQ DTCS kit, and is recommended for routine sequencing. The dGTP chemistry is recommended when customers cannot sequence through some difficult templates using dITP-based sequencing chemistries: Quick Start Kit and previous CEQ DTCS kit.

Note: Due to band compressions, we do not recommend using dGTP chemistry for routine sequencing. The dGTP chemistry is recommended only for sequencing through difficult regions that may include polymerase hard stops, secondary structures and GC rich regions. The dITP chemistry should be used to confirm all band compression regions and the regions adjacent to band compression. The quality values and quality scores available for analyzed data are tuned for the dITP chemistry, and may not accurately estimate the data quality of the dGTP chemistry.

Material Required

Materials provided by Beckman Coulter:
Methods Development Kit (P/N 608000):
- DNA polymerase
- Dye Terminators (ddUTP, ddGTP, ddCTP, ddATP)
- dNTP(I) Mix Solution
- dNTP(G) Mix Solution
- Sequencing Reaction Buffer
- pUC18 Control Template (0.25 µg/µL)
- M13 -47 Sequencing Primer (1.6 pmol/µL or 1.6 µM)
- Glycogen (20 mg/mL)
- Mineral Oil
- Sample Loading Solution (SLS)

Required materials not provided by Beckman Coulter:
- Molecular Biology Grade: Sterile dH_2O, 95% (v/v) ethanol/dH_2O, 70% (v/v) ethanol/ dH_2O
- 3M Sodium Acetate pH 5.2 - Sigma, Cat # 430771
- 100 mM Na_2-EDTA pH 8.0 (diluted from 0.5M Na_2-EDTA pH 8.0 - Sigma, Cat # 7889)
- Sterile tubes, 0.5 mL microfuge, 0.2 mL thin-wall thermal cycling tubes or plates
- Thermal cycler with heated lid

4300 N. Harbor Blvd., Fullerton, CA 92834-3100
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CAUTION

DNA polymerase is in a 50% glycerol solution. Pipet this solution slowly and carefully. The viscosity of the glycerol in the enzyme solution can lead to pipetting errors.

Preparation and Storage

Preparation and Storage of the Kit:
Storage of the Methods Development kit must be in a -20°C non-frost-free freezer.

Preparation and Storage of the Premix:
1. Prepare each Premix in a sterile 1.5 microfuge tube:

Component	dITP Chemistry	dGTP Chemistry
10X Sequencing Reaction Buffer	200 µL	200 µL
dNTP Mix	100 µL	100 µL
ddUTP Dye Terminator	200 µL	200 µL
ddGTP Dye Terminator	**100 µL**	**400 µL**
ddCTP Dye Terminator	200 µL	200 µL
ddATP Dye Terminator	200 µL	200 µL
Polymerase Enzyme	100 µL	100 µL
Total Volume	1100 µL	1400 µL

2. Mix and aliquot the Premix into sterile 0.5 mL microfuge tubes:

Component	dITP Chemistry	dGTP Chemistry
16-Sample Premix Aliquot	180 µL	230 µL

Each aliquot is enough for 16 samples.

3. Store the aliquots in a -20°C non-frost-free freezer. Minimize freezing and thawing of the aliquoted Premix.

Preparation of the DNA sequencing reaction*:

Prepare the 20 µL sequencing reaction in a 0.2 mL thin-wall tube or microplate well. Keep all reagents on ice while preparing the sequencing reactions and add components in the order listed below.

Component	dITP Chemistry	dGTP Chemistry
H_2O (to adjust total volume to 20 µL)	x.x µL	x.x µL
DNA Template† (See Template Preparation)	0.5 - 7.0 µL	0.5 - 4.0 µL
Customer supplied or -47 Sequencing Primer (1.6 pmol/µL or 1.6µM)	2.0 µL	2.0 µL
Premix	11.0 µL	14.0 µL
Total Volume	20.0 µL	20.0 µL

†Use 0.5 µL for pUC18 control template.

**Note: Mix reaction components thoroughly. Consolidate the liquid to the bottom of the tube or well by briefly centrifuging before thermal cycling.*

Thermal cycling programs:

dITP Chemistry:		dGTP Chemistry:	
96°C	20 sec.	96°C	20 sec.
50°C	20 sec.	50-68°C	20 sec.**
60°C	4 min.	68°C	2 min.

for 30 cycles followed by holding at 4°C

**For the supplied M13 -47 primer, an annealing temperature of 58°C is suitable for most templates. The thermal cycling parameters may need to be modified for other primer and template combinations. For the annealing step, a temperature based on the primer melting temperature (T_m) minus 3 to 5°C is recommended as a starting point.

Ethanol precipitation:

1. Prepare a labeled, sterile 0.5 mL microfuge tube for each sample.
2. Prepare fresh Stop Solution/Glycogen mixture as follows (per sequencing reaction): 2 µL of 3M Sodium Acetate (pH 5.2), 2 µL of 100mM Na_2-EDTA (pH 8.0) and 1µL of 20 mg/mL of glycogen (supplied with the kit). To each of the labeled tubes, add 5 µL of the Stop Solution/Glycogen mixture. Transfer the sequencing reaction to the appropriately labeled 0.5 mL tube and mix thoroughly.
3. Add 60 µL cold 95% (v/v) ethanol/dH_2O from -20°C freezer and mix thoroughly. Immediately centrifuge at 14,000 rpm at 4°C for 15 minutes. Carefully remove the supernatant with a micropipette (the pellet should be visible).

   Note: For multiple samples, always add the cold ethanol/dH_2O immediately before centrifugation.
4. Rinse the pellet 2 times with 200 µL 70% (v/v) ethanol/dH_2O from -20°C freezer. For each rinse, centrifuge immediately at 14,000 rpm at 4°C for a minimum of 2 minutes. After centrifugation carefully remove all of the supernatant with a micropipette.
5. Vacuum dry for 10 minutes (or until dry).
6. Resuspend the sample in 40 µL of the Sample Loading Solution (provided in the kit). See Appendix C for handling and storage of the Sample Loading Solution.

   Note: For plate precipitation instructions, refer to the Applications Information Bulletin (A1903A), A Rapid and Efficient Method for the Post-Reaction Clean Up of Labeled Dye Terminator Sequencing Products.

Sample preparation for loading into the instrument:

1. Transfer the resuspended samples to the appropriate wells of the polypropylene sample plate recommended for the instrument.
2. Overlay each of the resuspended samples with one drop of light mineral oil (provided in the kit).
3. Load the sample plate into the instrument and start the desired method.

   Note: When sequencing with dGTP chemistry, the capillary temperature of the separation method used on the CEQ Genetic Analysis System may be increased to reduce some band compressions.

Appendix

Appendix A

Sequencing of PCR products

All PCR products must be homogeneous in size as judged by gel electrophoresis.

Purified PCR products

- Remove unincorporated primers and dNTPs using QIAGEN QIAquick™ PCR purification system. Alternatively, unicorporated primers and dNTPs can be removed by Exo-SAP digestion using USB ExoSAP-IT®, followed by ethanol precipitation.
- Use 25-100 fmoles of PCR and 3.2 pmoles of primer.

Unpurified PCR products

- For the original PCR amplification, the primer concentration should be 0.2 µM or less, while the dNTP concentration should be 50 µM or less.
- The amplification should be sufficient to produce a concentration of amplified fragment that is >10 fmoles/µL.
- Dilute this amplified fragment approximately 10 fold to result in a concentration of >1 fmol/µL.
- Use 5-15 fmoles of this diluted, unpurified PCR product and 3.2 pmoles of primer.

Appendix B

Sequencing of Large Templates

Adding 50-100 fmol for large templates such as BACs, cosmids and PACs is impractical. The following procedure should be used when sequencing large templates.

1. Use 1.5 µg of the template in 6 µL of deionized water.
2. Pre-heat the template at 96°C for 1 minute. See Template Pre-Heat Treatment for details.
3. Add the sequencing reaction components as described in the standard protocol.
4. Cycle for 50 cycles using the appropriate cycling conditions for the primer being used.
5. Ethanol precipitate, as normal.

Appendix C

- Store the Sample Loading Solution in 350 µL aliquots at -20°C in a non-frost-free freezer.
- Use each aliquot only once. Do not freeze/thaw the Sample Loading Solution.

Appendix D

Optional SAP Treatment for dGTP samples

After thermal cycling is complete, an optional Shrimp Alkaline Phosphatase (SAP) treatment can be performed for removal of free dye terminator peaks as needed.

Add the following to each 20 µL of sequencing reaction:

2 µL	10x SAP Reaction Buffer*
1 µL	SAP (1 unit/µL)

Mix thoroughly by pipetting up and down. Consolidate the liquid to the bottom of the tube or well by briefly centrifuging before incubation.

*If 10x SAP reaction buffer is not available, replace the 2 µL of 10x SAP reaction buffer with 2 µL of 100 mM $MgCl_2$.

Incubation Conditions for SAP treatment:

37°C 30 min.
Followed by holding at 4°C

Continue to ethanol precipitation step.

If a SAP treatment is performed, modify the "Delay" setting on the "Initial Data Detection" tab of the "Sequencing Analysis Parameters Editor" in the sequencing analysis module to 0.1 minute. Alternatively, if a SAP treatment is not performed, but exclusion of free dye terminator peaks is desired, modify the "Delay" setting on the "Initial Data Detection" tab of the "Sequencing Analysis Parameters Editor" in the sequencing analysis module to 1.4 minutes.

Template Preparation

DNA Template Preparation:

Prepare sufficient template to allow for accurate quantitation and purity testing. Quality of the DNA template will depend upon the procedure and the source of the DNA used. The following are the recommended protocols:

- QIAGEN QIAwell™ and QIAprep™ DNA isolation protocols (dsDNA and ssDNA)
- QIAGEN QIAquick™ PCR purification protocol (PCR products) *

Note: Determine the quality and quantity of template DNA by agarose gel electrophoresis.

DNA Template Amount:

The amount of template DNA to use in the sequencing reaction depends on the form of the DNA (dsDNA plasmid, ssDNA, M13, PCR product, etc.). It is important to accurately quantitate the amount (moles) of DNA when performing the DNA sequencing reaction (see formula and table below for details). The molar ratio of primer to template must be ≥ 40:1. Listed below are the recommended amounts of DNA:

dsDNA	50-100 fmol
ssDNA	25-50 fmol
Purified PCR products	25-100 fmol

The following table can be used to estimate DNA concentrations.

Table for estimating the dsDNA concentration.**

Size (kilobase pairs)	ng for 25 fmol	ng for 50 fmol	ng for 100 fmol
0.2	3.3	6.5	13
0.3	4.9	9.8	20
0.4	6.5	13	26
0.5	8.1	16	33
1.0	16	33	65
2.0	33	65	130
3.0	50	100	195
4.0	65	130	260
5.0	80	165	325
6.0	100	195	390
8.0	130	260	520
10.0	165	325	650
12.0	195	390	780
14.0	230	455	910
16.0	260	520	1040
18.0	295	585	1170
20.0	325	650	1300
48.5	790	1500†	1500†

**For ssDNA, the values (ng) should be divided by 2.
† Do not use more than 1.5 µg of template DNA.

Template Pre-Heat Treatment

For certain plasmid DNA templates (not the included pUC18 control DNA), the following pre-heat treatment improves both signal strength and current stability.

Dilute the template to the appropriate concentration in water. Heat the template at 96°C for 1 minute in a thermal cycler and then cool to room temperature before adding the remainder of the sequencing-reaction components. Do not add any other sequencing-reaction components to the plasmid template before carrying out this pre-heat treatment. If the raw data signal declines steeply when using this treatment, change the heating conditions to 86°C for 5 minutes. If the current is low or unstable following this treatment, increase the treatment to 96°C for 3 minutes.

See the detailed Dye Terminator Cycle Sequencing Chemistry Protocol (P/N 718119) or (P/N 390003) for more information.

Handling Precautions

Please be aware of the handling precautions listed below. For detailed information, see 67-548-EEC (Directive on Dangerous Substances), 88-379-EEC (Dangerous Preparations Directive) and 21 CFR 1910.1200 (USA OSHA Hazard Communications).

Sample Loading Solution:
Toxic. Contains Formamide. R61 May cause harm to unborn child. R36/37 Irritating to eyes and respiratory system. S24/25 Avoid contact with skin and eyes. S37 Wear suitable gloves. S45 In case of accident, or if you feel unwell, seek medical advice immediately. S53 Avoid exposure-obtain special instructions before use.

Dye Terminators:
Contains <20% Methanol. R20/21/22 Harmful by inhalation, in contact with skin and if swallowed. R23/24/25 Toxic by inhalation, in contact with skin and if swallowed. R39 Danger of very serious irreversible effects. S36/37 Wear suitable protective clothing and gloves. S45 In case of accident or if you feel unwell, seek medical advice immediately. S60 This material and/or its container must be disposed of as hazardous waste. S7/9 Keep container tightly closed and in a well-ventilated place.

Appendix L: Sequence I Log

Samp ID New	ng/ul	DNA Templat Amount (	Water Amoun (ul)	Well #	Red w/fa	Colum b	Ro w	Date S
25	199.06	1.3	5.7	6		6	A	1
27	230.47	1.1	5.9	30		6	B	1
26	274.96	0.9	6.1	42		6	C	1
28	289.28	0.9	6.1	54		6	D	1
29	312.45	0.8	6.2	66		6	E	1
30	249.84	1.0	6.0	78	1	6	F	1
31	146.06	1.8	5.2	90		6	G	1
32	275.9	0.9	6.1	102		6	H	1

Appendix M: Sequence II Log

Sample ID New	ng/ul	DNA Template Amount (ul)	Water Amount (ul)	Well #	Redo w/fail	Colum b	Ro w	Date Seq
1	292.79	0.9	6.1	58		10	E	2
2	277.1	0.9	6.1	69		9	F	2
3	444.22	0.6	6.4	11	1	11	A	2
5	273.72	0.9	6.1	72	1	12	F	2
7	347.51	0.7	6.3	31		7	C	2
8	415.48	0.6	6.4	13	1	1	B	2
9	304.34	0.9	6.1	52		4	E	2
10	321.45	0.8	6.2	43		7	D	2
11	629.51	0.4	6.6	1	1	1	A	2
12	253.79	1.0	6.0	83	1	11	G	2
16	313.1	0.8	6.2	47		11	D	2
17	277.2	0.9	6.1	68		8	F	2
18	384.56	0.7	6.3	20		8	B	2
19	263.2	1.0	6.0	78		6	G	2
20	374.85	0.7	6.3	22	1	10	B	2
22	395.66	0.7	6.3	17	1	5	B	2
23	260.03	1.0	6.0	80		8	G	2
26	274.96	0.9	6.1	71		11	F	2
28	289.28	0.9	6.1	61	1	1	F	2
29	312.45	0.8	6.2	48	1	12	D	2
30	249.84	1.0	6.0	88		4	H	2
32	275.9	0.9	6.1	70	1	10	F	2
34	584.08	0.4	6.6	2	1	2	A	2
36	252.04	1.0	6.0	86	1	2	H	2
40	356.49	0.7	6.3	29	1	5	C	2
41	265.12	1.0	6.0	76		4	G	2
42	427.52	0.6	6.4	12		12	A	2
43	257.57	1.0	6.0	81		9	G	2
44	250.64	1.0	6.0	87		3	H	2
45	306.31	0.8	6.2	51		3	E	2
46	393.63	0.7	6.3	19		7	B	2
47	360	0.7	6.3	26	1	2	C	2
48	407.97	0.6	6.4	14		2	B	2
49	324.12	0.8	6.2	42		6	D	2
52	335.07	0.8	6.2	37	1	1	D	2
55	291.36	0.9	6.1	59		11	E	2
56	285.77	0.9	6.1	64		4	F	2
57	298.27	0.9	6.1	55		7	E	2
58	263.66	1.0	6.0	77		5	G	2
60	245.36	1.1	5.9	91		7	H	2
61	262.73	1.0	6.0	79		7	G	2
62	310.7	0.8	6.2	50		2	E	2
63	296.8	0.9	6.1	56		8	E	2
64	315.25	0.8	6.2	46		10	D	2
65	272.85	1.0	6.0	73	1	1	G	2
66	345.22	0.8	6.2	32		8	C	2
67	296.31	0.9	6.1	57	1	9	E	2
68	281.54	0.9	6.1	67		7	F	2
70	282.55	0.9	6.1	66		6	F	2
73	288.72	0.9	6.1	62		2	F	2
74	289.64	0.9	6.1	60		12	E	2
75	304.29	0.9	6.1	53		5	E	2
83	355.84	0.7	6.3	30		6	C	2
84	243.21	1.1	5.9	94		12	H	2
89	243.22	1.1	5.9	93		9	H	2
92	246.08	1.1	5.9	89		5	H	2
93	253.34	1.0	6.0	85	1	1	H	2
94	243.56	1.1	5.9	92		8	H	2
97	340.17	0.8	6.2	34		10	C	2
99	334.25	0.8	6.2	38		2	D	2
103	357.91	0.7	6.3	27		3	C	2
104	253.44	1.0	6.0	84		12	G	2
105	241.61	1.1	5.9	95		11	H	2
106	406	0.6	6.4	15	1	3	B	2
107	329.32	0.8	6.2	41		5	D	2
109	238.06	1.1	5.9	96		10	H	2
110	300.52	0.9	6.1	54		6	E	2
111	337.11	0.8	6.2	35		11	C	2
112	311.38	0.8	6.2	49	1	1	E	2
113	334.18	0.8	6.2	39		3	D	2
114	471.8	0.6	6.4	7		7	A	2
115	395.22	0.7	6.3	18	1	6	B	2
117	341.55	0.8	6.2	33		9	C	2
118	333.5	0.8	6.2	40		4	D	2
119	382.27	0.7	6.3	21		9	B	2
120	495.51	0.5	6.5	6		6	A	2
122	271.97	1.0	6.0	74		2	G	2
123	363.62	0.7	6.3	25	1	1	C	2
124	458.39	0.6	6.4	9	1	9	A	2
126	255.24	1.0	6.0	82		10	G	2
127	539.81	0.5	6.5	4		4	A	2
128	569.72	0.5	6.5	3		3	A	2
129	363.84	0.7	6.3	24	1	12	B	2
130	401.15	0.6	6.4	16	1	4	B	2
131	268.96	1.0	6.0	75		3	G	2
132	335.22	0.8	6.2	36		12	C	2
133	463.77	0.6	6.4	8	1	8	A	2
134	356.68	0.7	6.3	28		4	C	2
135	320.61	0.8	6.2	44		8	D	2
136	454.61	0.6	6.4	10		10	A	2
137	369.53	0.7	6.3	23	1	11	B	2
139	498.32	0.5	6.5	5		5	A	2
140	245.61	1.1	5.9	90		6	H	2
141	286.04	0.9	6.1	63		3	F	2
142	283.69	0.9	6.1	65		5	F	2
143	317.1	0.8	6.2	45		9	D	2

Appendix N: Sequence III Log

Sample ID New	ng/ul	DNA Template Amount (ul)	Water Amount (ul)	Well #	Redow / fail	Columb	Row	Date Seq
3	n/a	0.6	6.4	10		10	A	3
5	n/a	0.9	6.1	83		11	G	3
8	n/a	0.6	6.4	20		8	B	3
12	n/a	1.0	6.0	89		5	H	3
20	n/a	0.7	6.3	46		10	D	3
22	n/a	0.7	6.3	29		5	C	3
25	n/a	1.3	5.7	96		12	H	3
27	n/a	1.1	5.9	95		11	H	3
28	n/a	0.9	6.1	80		8	G	3
29	n/a	0.8	6.2	77		5	G	3
32	n/a	0.9	6.1	82		10	G	3
34	n/a	0.4	6.6	2		2	A	3
36	n/a	1.0	6.0	91		7	H	3
40	n/a	0.7	6.3	63		3	F	3
47	n/a	0.7	6.3	59		11	E	3
52	n/a	0.8	6.2	69		9	F	3
65	n/a	1.0	6.0	84		12	G	3
67	n/a	0.9	6.1	79		7	G	3
93	n/a	1.0	6.0	90		6	H	3
106	n/a	0.6	6.4	22		10	B	3
112	n/a	0.8	6.2	78		6	G	3
115	n/a	0.7	6.3	30		6	C	3
123	n/a	0.7	6.3	57		9	E	3
124	n/a	0.6	6.4	7		7	A	3
129	n/a	0.7	6.3	56		8	E	3
130	n/a	0.6	6.4	26		2	C	3
133	n/a	0.6	6.4	6		6	A	3
137	n/a	0.7	6.3	54		6	E	3
160	417.99	0.6	6.4	17		5	B	3
161	393.29	0.7	6.3	32		8	C	3
162	512.57	0.5	6.5	3		3	A	3
163	421.42	0.6	6.4	15		3	B	3
164	409.55	0.6	6.4	21		9	B	3
165	375.59	0.7	6.3	44		8	D	3
166	416.67	0.6	6.4	19		7	B	3
167	444.24	0.6	6.4	9		9	A	3
168	374.55	0.7	6.3	47		11	D	3
169	399.46	0.7	6.3	27		3	C	3
170	445.99	0.6	6.4	8		8	A	3
171	270.15	1.0	6.0	86		2	H	3
172	427.66	0.6	6.4	14		2	B	3
173	343.48	0.8	6.2	68		8	F	3
174	405	0.6	6.4	23		11	B	3
175	374.01	0.7	6.3	48		12	D	3
176	484.4	0.5	6.5	5		5	A	3
177	486.16	0.5	6.5	4		4	A	3
178	434.82	0.6	6.4	12		12	A	3
179	348.62	0.7	6.3	65		5	F	3
180	436.9	0.6	6.4	11		11	A	3
181	256.02	1.0	6.0	88		4	H	3
182	372.44	0.7	6.3	50		2	E	3
183	371.92	0.7	6.3	51		3	E	3
184	395.02	0.7	6.3	31		7	C	3
185	392.27	0.7	6.3	33		9	C	3
186	378.66	0.7	6.3	41		5	D	3
187	417.35	0.6	6.4	18		6	B	3
188	370.95	0.7	6.3	53		5	E	3
189	324.44	0.8	6.2	72		12	F	3
190	345.18	0.8	6.2	67		7	F	3
191	383.31	0.7	6.3	40		4	D	3
192	389.07	0.7	6.3	36		12	C	3
193	389.56	0.7	6.3	35		11	C	3
194	257.88	1.0	6.0	87		3	H	3
195	348.19	0.7	6.3	66		6	F	3
196	399.33	0.7	6.3	28		4	C	3
197	357.2	0.7	6.3	62		2	F	3
198	403.84	0.6	6.4	24		12	B	3
199	419.81	0.6	6.4	16		4	B	3
200	331.5	0.8	6.2	70		10	F	3
201	387.85	0.7	6.3	38		2	D	3
202	391.69	0.7	6.3	34		10	C	3
203	375.03	0.7	6.3	45		9	D	3
204	285.98	0.9	6.1	81		9	G	3
205	377.05	0.7	6.3	43		7	D	3
206	362.82	0.7	6.3	58		10	E	3
207	357.35	0.7	6.3	60		12	E	3
208	349.78	0.7	6.3	64		4	F	3
209	232.44	1.1	5.9	93		9	H	3
210	371.3	0.7	6.3	52		4	E	3
211	320.58	0.8	6.2	75		3	G	3
212	377.2	0.7	6.3	42		6	D	3
213	328.25	0.8	6.2	71		11	F	3
214	384.78	0.7	6.3	39		3	D	3
215	313.14	0.8	6.2	76		4	G	3
216	321.59	0.8	6.2	74		2	G	3
217	229.59	1.1	5.9	94		10	H	3
218	243.1	1.1	5.9	92		8	H	3
219	367.91	0.7	6.3	55		7	E	3
[redacted]				1		1	A	3
				13		1	B	3
				25		1	C	3
				37		1	D	3
				49		1	E	3
				61		1	F	3
				73		1	G	3
				85		1	H	3

Appendix O: Sequence IV Log

Sample ID New	ng/ul	DNA Template Amount (ul)	Water Amount (ul)	Well #	Redow /fail	Columb	Row	Date Seq
4	213.17	1.2	5.8	76		4	G	4
21	210.02	1.2	5.8	79		7	G	4
31	146.06	1.8	5.2	95		11	H	4
35	192.92	1.3	5.7	82		10	G	4
38	200.24	1.3	5.7	81		9	G	4
39	228.77	1.1	5.9	64		4	F	4
50	178.78	1.5	5.5	88		4	H	4
51	144.96	1.8	5.2	96		12	H	4
54	192.85	1.3	5.7	83		11	G	4
59	237.63	1.1	5.9	55		7	E	4
69	228.65	1.1	5.9	65		5	F	4
71	157.68	1.6	5.4	93		9	H	4
72	223.3	1.2	5.8	70		10	F	4
76	171.72	1.5	5.5	91		7	H	4
78	228.24	1.1	5.9	66		6	F	4
79	161.69	1.6	5.4	92		8	H	4
81	227.68	1.1	5.9	67		7	F	4
82	234.96	1.1	5.9	58		10	E	4
86	220.79	1.2	5.8	71		11	F	4
88	212.89	1.2	5.8	77		5	G	4
90	235.19	1.1	5.9	57		9	E	4
91	171.78	1.5	5.5	90		6	H	4
95	173.77	1.5	5.5	89		5	H	4
96	155.33	1.7	5.3	94		10	H	4
101	231.8	1.1	5.9	62		2	F	4
102	192.25	1.4	5.6	84		12	G	4
108	180.31	1.4	5.6	86		2	H	4
116	227.13	1.1	5.9	69		9	F	4
121	228.78	1.1	5.9	63		3	F	4
125	220.19	1.2	5.8	72		12	F	4
139	184.11	1.4	5.6	85		1	H	4
144	235.23	1.1	5.9	56		8	E	4
222	282.64	0.9	6.1	45		9	D	4
223	337.47	0.8	6.2	30		6	C	4
224	374.43	0.7	6.3	23		11	B	4
225	272.7	1.0	6.0	48		12	D	4
226	447.01	0.6	6.4	3		3	A	4
228	278.6	0.9	6.1	46		10	D	4
233	180.13	1.4	5.6	87		3	H	4
234	475.59	0.5	6.5	1		1	A	4
235	397.65	0.7	6.3	17		5	B	4
237	287.57	0.9	6.1	43		7	D	4
238	253.66	1.0	6.0	53		5	E	4
240	306.66	0.8	6.2	36		12	C	4
241	259.76	1.0	6.0	51		3	E	4
242	218.2	1.2	5.8	73		1	G	4
243	294.69	0.9	6.1	41		5	D	4
244	212.23	1.2	5.8	78		6	G	4
245	304.98	0.9	6.1	39		3	D	4
246	367.74	0.7	6.3	25		1	C	4
247	205.32	1.3	5.7	80		8	G	4
248	232.2	1.1	5.9	60		12	E	4
249	215.89	1.2	5.8	74		2	G	4
250	214.74	1.2	5.8	75		3	G	4
251	232.16	1.1	5.9	61		1	F	4
253	258.15	1.0	6.0	52		4	E	4
254	287.66	0.9	6.1	42		6	D	4
255	265.66	1.0	6.0	50		2	E	4
256	295.54	0.9	6.1	40		4	D	4
257	317.79	0.8	6.2	35		11	C	4
258	306.33	0.8	6.2	38		2	D	4
259	327.32	0.8	6.2	33		9	C	4
260	266.28	1.0	6.0	49		1	E	4
261	343.72	0.8	6.2	29		5	C	4
262	227.67	1.1	5.9	68		8	F	4
263	283.48	0.9	6.1	44		8	D	4
264	234.42	1.1	5.9	59		11	E	4
265	321.58	0.8	6.2	34		10	C	4
266	334.87	0.8	6.2	32		8	C	4
267	306.38	0.8	6.2	37		1	D	4
268	241.97	1.1	5.9	54		6	E	4
269	362.94	0.7	6.3	26		2	C	4
270	277.5	0.9	6.1	47		11	D	4
271	381.27	0.7	6.3	21		9	B	4
272	353.52	0.7	6.3	27		3	C	4
273	426.6	0.6	6.4	8		8	A	4
274	337.38	0.8	6.2	31		7	C	4
275	413.8	0.6	6.4	13		1	B	4
276	391.65	0.7	6.3	18		6	B	4
277	381.23	0.7	6.3	22		10	B	4
278	383.81	0.7	6.3	19		7	B	4
279	400.11	0.6	6.4	16		4	B	4
280	423.41	0.6	6.4	9		9	A	4
281	403.86	0.6	6.4	14		2	B	4
282	371.03	0.7	6.3	24		12	B	4
283	402.1	0.6	6.4	15		3	B	4
284	418.64	0.6	6.4	11		11	A	4
285	428.92	0.6	6.4	6		6	A	4
286	382.73	0.7	6.3	20		8	B	4
287	419.06	0.6	6.4	10		10	A	4
288	417.48	0.6	6.4	12		12	A	4
289	428.9	0.6	6.4	7		7	A	4
290	431.72	0.6	6.4	4		4	A	4
291	454.59	0.6	6.4	2		2	A	4
292	350.84	0.7	6.3	28		4	C	4
293	430.75	0.6	6.4	5		5	A	4

Appendix P: Sequence V Log

Sample ID	ng/ul	Template DNA (ul)	H2O (ul)	Well ID	Redow / fail	Columb	Row	Date Seq
299	509.6	0.5	6.5	1		1	A	5
294	498.63	0.5	6.5	2		2	A	5
314	479.87	0.5	6.5	3		3	A	5
315	475.43	0.5	6.5	4		4	A	5
295	467.62	0.6	6.4	5		5	A	5
297	453.09	0.6	6.4	6		6	A	5
298	450.19	0.6	6.4	7		7	A	5
326	446.42	0.6	6.4	8		8	A	5
310	441.46	0.6	6.4	9		9	A	5
322	439.28	0.6	6.4	10		10	A	5
311	438.95	0.6	6.4	11		11	A	5
321	435.9	0.6	6.4	12		12	A	5
303	434.59	0.6	6.4	13		1	B	5
323	428.21	0.6	6.4	14		2	B	5
318	423.29	0.6	6.4	15		3	B	5
306	413.19	0.6	6.4	16		4	B	5
319	410.8	0.6	6.4	17		5	B	5
320	409.1	0.6	6.4	18		6	B	5
300	400.92	0.6	6.4	19		7	B	5
304	400.7	0.6	6.4	20		8	B	5
316	397.16	0.7	6.3	21		9	B	5
330	392.99	0.7	6.3	22		10	B	5
313	392.33	0.7	6.3	23		11	B	5
301	390.21	0.7	6.3	24		12	B	5
296	388.46	0.7	6.3	25		1	C	5
317	387.75	0.7	6.3	26		2	C	5
325	384.77	0.7	6.3	27		3	C	5
324	381.56	0.7	6.3	28		4	C	5
327	380.26	0.7	6.3	29		5	C	5
328	372.47	0.7	6.3	30		6	C	5
329	369.08	0.7	6.3	31		7	C	5
308	356.71	0.7	6.3	32		8	C	5
305	355.5	0.7	6.3	33		9	C	5
312	352.74	0.7	6.3	34		10	C	5
344	351.01	0.7	6.3	35		11	C	5
309	348.96	0.7	6.3	36		12	C	5
307	348.16	0.7	6.3	37		1	D	5
332	344.13	0.8	6.2	38		2	D	5
302	339.23	0.8	6.2	39		3	D	5
333	326.06	0.8	6.2	40		4	D	5
393	319.67	0.8	6.2	41		5	D	5
355	316.7	0.8	6.2	42		6	D	5
360	314.95	0.8	6.2	43		7	D	5
351	311.18	0.8	6.2	44		8	D	5
358	307.72	0.8	6.2	45		9	D	5
350	307.51	0.8	6.2	46		10	D	5
366	306.92	0.8	6.2	47		11	D	5
354	297.13	0.9	6.1	48		12	D	5
340	292.26	0.9	6.1	49		1	E	5
349	291.7	0.9	6.1	50		2	E	5
390	290.81	0.9	6.1	51		3	E	5
369	283.48	0.9	6.1	52		4	E	5
378	278.86	0.9	6.1	53		5	E	5
338	276.29	0.9	6.1	54		6	E	5
352	275.41	0.9	6.1	55		7	E	5
381	272.29	1.0	6.0	56		8	E	5
336	272.25	1.0	6.0	57		9	E	5
382	272.04	1.0	6.0	58		10	E	5
372	271.51	1.0	6.0	59		11	E	5
361	269.11	1.0	6.0	60		12	E	5
334	268.08	1.0	6.0	61		1	F	5
364	267.65	1.0	6.0	62		2	F	5
376	264.48	1.0	6.0	63		3	F	5
353	262.58	1.0	6.0	64		4	F	5
337	259.43	1.0	6.0	65		5	F	5
375	257.96	1.0	6.0	66		6	F	5
356	257.23	1.0	6.0	67		7	F	5
379	257.22	1.0	6.0	68		8	F	5
385	256.73	1.0	6.0	69		9	F	5
335	255.75	1.0	6.0	70		10	F	5
377	255.65	1.0	6.0	71		11	F	5
387	254.55	1.0	6.0	72		12	F	5
374	253.79	1.0	6.0	73		1	G	5
373	252.95	1.0	6.0	74		2	G	5
362	250.21	1.0	6.0	75		3	G	5
368	249.67	1.0	6.0	76		4	G	5
384	249.52	1.0	6.0	77		5	G	5
389	247.48	1.1	5.9	78		6	G	5
363	245.42	1.1	5.9	79		7	G	5
342	242.15	1.1	5.9	80		8	G	5
370	241.39	1.1	5.9	81		9	G	5
331	239.49	1.1	5.9	82		10	G	5
357	239.27	1.1	5.9	83		11	G	5
339	236.6	1.1	5.9	84		12	G	5
371	235.35	1.1	5.9	85		1	H	5
392	235.06	1.1	5.9	86		2	H	5
380	230.68	1.1	5.9	87		3	H	5
359	223.76	1.2	5.8	88		4	H	5
386	222.21	1.2	5.8	89		5	H	5
367	221.63	1.2	5.8	90		6	H	5
365	220.55	1.2	5.8	91		7	H	5
395	214.1	1.2	5.8	92		8	H	5
396	211.51	1.2	5.8	93		9	H	5
383	210.82	1.2	5.8	94		10	H	5
394	206.39	1.3	5.7	95		11	H	5
388	186.67	1.4	5.6	96		12	H	5
341	176.12	1.5	5.5					
343	173.29	1.5	5.5					
391	172.8	1.5	5.5					
345	31.28	8.3	-1.3					
346	2.68	97.0	-90.0					
347	1.77	146.9	-139.9					
348	1.24	209.7	-202.7					

Appendix Q: Sequence VI Log

Sam ID	ng/ul	Templ DNA (	H2O (u	Well	Redov ID il	Row	Column	Dat Se
414	296.77	0.9	6.1	1		A	8	6
418	289.85	0.9	6.1	2		B	8	6
417	289.03	0.9	6.1	3		C	8	6
410	287.53	0.9	6.1	4		D	8	6
419	285.2	0.9	6.1	5		E	8	6
416	280.23	0.9	6.1	6		F	8	6
409	267.58	1.0	6.0	7		G	8	6
402	267.27	1.0	6.0	8		H	8	6
415	265.05	1.0	6.0	9		A	9	6
420	258.77	1.0	6.0	10		B	9	6
412	244.8	1.1	5.9	11		C	9	6
422	242.8	1.1	5.9	12		D	9	6
421	240.39	1.1	5.9	13		E	9	6
413	237.9	1.1	5.9	14		F	9	6
401	236.06	1.1	5.9	15		G	9	6
403	230	1.1	5.9	16		H	9	6
386	222.21	1.2	5.8	17		A	10	6
367	221.63	1.2	5.8	18		B	10	6
365	220.55	1.2	5.8	19		C	10	6
400	218.82	1.2	5.8	20		D	10	6
395	214.1	1.2	5.8	21		E	10	6
399	214.09	1.2	5.8	22		F	10	6
398	212.92	1.2	5.8	23		G	10	6
396	211.51	1.2	5.8	24		H	10	6
383	210.82	1.2	5.8	25		A	11	6
394	206.39	1.3	5.7	26		B	11	6
405	204.74	1.3	5.7	27		C	11	6
406	201.09	1.3	5.7	28		D	11	6
407	200.95	1.3	5.7	29		E	11	6
404	194.18	1.3	5.7	30		F	11	6
408	188.83	1.4	5.6	31		G	11	6
388	186.67	1.4	5.6	32		H	11	6
423	178.61	1.5	5.5	33		A	12	6
341	176.12	1.5	5.5	34		B	12	6
343	173.29	1.5	5.5	35		C	12	6
391	172.8	1.5	5.5	36		D	12	6
411	167.04	1.6	5.4	37		E	12	6
397	165.88	1.6	5.4	38		F	12	6
424	6.48	40.1	-33.1	[redacted]				
426	3.68	70.7	-63.7					
425	2.9	89.7	-82.7					
427	1.63	159.5	-152.					
429	1.58	164.6	-157.					
428	1.22	213.1	-206.					

Appendix R: Sequence I Output (FASTA Format)

SEQUENCE I

```
>25.A06_060110223U    671      0    671   CEQ
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TTTCCCCCATTGCGCAAAATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGTAC
ACCCTCTCAGGCCGGCTACCCATCATAACCTTGGTAGGCCATTACCCTACCAACTAGCTAATAGGACGCAGGCTCATCC
TCAAGCGCCTTTCGGCTTTGACTCCGAAGACATAAGTCCCGGAGTTTCATGCGGTATTAGGCCCCCTTTCGAGGGTTTA
TTCCTCACTCGAGGGGAGATTACCCACGTGTTACTCACCCGTTCGCCGCTATTCCAAGGTATTGCTACCCTGAAACCGC
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>26.B06_060110223U    648      0    648   CEQ
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GGACGAGATTTTTCGTTGTAGCAATACGATGGAAAGTCTAGTGGCGAACGGGTGCTTAACACGTGGGCAACCTGCCGAG
AAGTGGGGGATAGCTCGCCGAAAGGCGAATTAATACCGCATGTGATTAGAGAGGACATCCTTTCGAAATTAAAGTCGGG
GGCAACCTGACGCTTTTTGTGGGGCCCGCGGGCCTATCGCTAGTTGGTGAGGTAGGTAAACGGGCTCAACCAAAAAGGC
TANGAATGATTCTAAAGGGGGTTTCCTGAAAGAAGAGGATGAAATCCCCCCCAACCACCCTGGGGTAACCTGGAGAAAC
AACCGGAACCAAGAACTCCCCTACCGGGGAAGGCAGCCAGTTAAAGGAATAATTGGGTCAAATGGGCGCAAGCCTGAAC
CAGCCATGCCGCGTGCAAGAAGACCGCCTTATTGGGTTGTAAAACTGCTTTTTATAAAAAAAGGAATGAAAACCCCCCC
TCCTCCGGCTGGAAGA

>27.C06_060110223U    678      0    678   CEQ
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TCAAGCGATCCCGCCATTCATATAGCAATATATGAAAGGCGGGATAGCGGCAAACGGGTTAGTAACGCGTAGATAACTT
ACCCCGAAGACTTGGATAACCCCGCGAAAGTGGGGCTAATACAAGATGGAAATGAGGGGACATATGTCATTCTCATTTA
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TATGACGGGTAGGGGGACTGAGAGGTTGGCCCCCAACATCGGAACTGAGACACTGTCCGGACTCCTACGGGAGGCTGCA
GTCAAGAACCTTCGACAATGGGCGAAAGCCTGATCGAGCGACGCCGCGTGACTGAAGAAGCTCTTCCGAATGGGTAAAT
GTCTTATATAGGGGGAGCAATTTTTGAGTGTACTCTATGAATAAGGGGTTCCTAAACTCGTGCAGCAGCCCGGTCAAGG
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>28.D06_060110223U    669      0    669   CEQ
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ACCGGATTGTTCACCCGGCCCTTATTCGTCCCTCGCGACAGTGCTTTACGATCCGAAGACCTTCATCACACACGCGGCG
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CAGTGTGGCTGATCGTCCTCTCAGACCAGCTACCCGTCGTAGCCTTGGTGGGCCATTACCCCGCCAACTAGCTGATGGG
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>29.E06_060110223U    440      0    440   CEQ
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GCAANGAGTNGCGTGGGGGGGGAAAATAACCACTTGGGCTCCGCAGAAAAGAGGGGAATCGAGCTCATAATATTCCCCC
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>30.F06_060110223U      0      0      0   CEQ
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>31.G06_060110223U 715 0 715 CEQ
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CGTCGTGGGTGAAAGAGCTTTACAACCCTAAGGCCTTCCTCACTCACGCGGCATTGCTGGATCAGGCTTGCGCCCATTG
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CGCCAGCGTTCGTTCTGAGCCAGGATCAAACTCTAAGGGCGAATCCAGCACACTGGCGGCCGTTACTAGTGGATCGAGC
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ACAC

>32.H06_060110223U 724 0 724 CEQ
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GGTCAATGGGCGCAAGCCTGAACCAGCCATCCCGCGTGAAGGAAGAAGGCGCTATGCGTTGTAAACTTCTTTTCCAGGG
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CAAAGGGCGATTCCAGCACACTGGCCGCCGTTTCTAGTGGATCCGAGCTCGGTACCCGCCTGGCGTATCCTTGGTCCTA
CTGTCCCGTGTTT

Appendix S: Sequence II Output (FASTA Format)

SEQUENCE II

```
>1.E10_06012710UA    669     22    669   CEQ
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TCGACCGTAGTCTTTCGTCCCTCCCCACAGAGCTTTACAACCCGAAGGCCTTCATCACTCACGCGGCGTCGCTGGTTCAGGCTTGCGCC
CATTGACCAAAGATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCCCAGTACCAGTGTGGCTGATCATCCTCTCAGACCAGC
TACCCGTCTTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTGATGGGCCGCAGACCCCTCCTCTGACGACTGGCCGTTGCCGGTCCC
AGCCTTTGTCCGCAAATACCTTGGTACTCGCGGCGTTATGCGGTATTAGCGCTCCTTTCGGAACGTTATCCCCCTTCGAGGGCAGGTTA
TCTACGTGTTACGCCCCCGTGCGCACTTACTAACGGTGCCCCTTCGCGTGCACTGCTGTGTANCCCGCTACGTCCCGACAGACACCAAG
CATCAACGCGGATTACACCGTACGCGCATGTTCTCTTAGTCACAAT

>2.F09_06012710UJ    738      0    738   CEQ
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CGCTATGCGTTGTAAACTTCTTTTCCAGAGGAATAAAATTTTGTACGAGTACAGAACTGAAAGTACTCTGGGAATAAGCATCGGCTAAC
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>3.A11_06012710UP    935      0    935   CEQ
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CTGCGGCTTTTCTGTCTTGGCGGGTATNCGGTCTTTCTGTCTCCT

>5.F12_06012710UQ    568      0    568   CEQ
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>8.B01_06012710UT      5      5      5   CEQ
TCTAC

>9.E04_06012710UU    685      0    685   CEQ
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>10.D07_06012710UB 789 0 789 CEQ
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>12.G11_06012710UD 768 107 768 CEQ
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>16.D11_06012710UF 761 0 761 CEQ
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>17.F08_06012710UG 761 51 761 CEQ
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>19.G06_06012710UH 774 0 774 CEQ
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>20.B10_06012710UK 1025 0 1025 CEQ
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>22.B05_06012710UM 867 0 867 CEQ
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>23.G08_06012710UN 738 6 738 CEQ
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Appendix T: Sequence III Output (FASTA Format)

SEQUENCE III

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>204.G09_06012616BZ 713 0 713 CEQ
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>205.D07_06012616C0 725 0 725 CEQ
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>206.E10_06012616C1 231 35 231 CEQ
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>207.E12_06012616C3 568 0 568 CEQ
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>208.F04_06012616C4 726 0 726 CEQ
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>212.D06_06012616C8 799 0 799 CEQ
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>215.G04_06012616CC 776 0 776 CEQ
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>217.H10_06012616CE 834 0 834 CEQ
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>218.H08_06012616CF 791 6 791 CEQ
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Appendix U: Sequence IV Output (FASTA Format)

SEQUENCE IV

>4.G04_06012620EX 725 0 725 CEQ
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>31.H11_06012620ES 724 0 724 CEQ
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>35.G10_06012620ET 743 0 743 CEQ
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>39.F04_06012620EW 735 0 735 CEQ
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Appendix V: Sequence V Output (FASTA Format)

SEQUENCE V

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>377.F11_06012616IB 705 0 705 CEQ
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>378.E05_06012616IC 709 0 709 CEQ
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>381.E08_06012616IF 732 0 732 CEQ
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>382.E10_06012616IH 644 0 644 CEQ
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>384.G05_06012616II 710 3 710 CEQ
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>389.G06_06012616IM 691 0 691 CEQ
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>390.E03_06012616IN 745 0 745 CEQ
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>393.D05_06012616IP 655 0 655 CEQ
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Appendix W: Sequence VI Output (FASTA Format)

SEQUENCE VI

>423.A12_06013023GT 670 0 670 CEQ
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>341.B12_06013023GT 700 0 700 CEQ
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>343.C12_06013023GT 691 0 691 CEQ
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>365.C10_0601302074 740 0 740 CEQ
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>367.B10_0601302074 676 4 676 CEQ
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Appendix X: BLAST Version ID Definitions

Version	DEFINITION	SOURCE	isolation_source
AM086107	Uncultured bacterium partial 16S rRNA gene, clone c5LKS6	uncultured bacterium	lake profundal sediment
AM086108	Uncultured bacterium partial 16S rRNA gene, clone c5LKS7	uncultured bacterium	lake profundal sediment
AM180059	Uncultured bacterium partial 16S rRNA gene, clone A1-632	uncultured bacterium	Reactor for nitrogen and phosphous removal
AY214753	Uncultured candidate division OP11 bacterium clone BB-1-F3 16S ribosomal RNA gene, partial sequence	uncultured candidate division OP11 bacterium	soil
AY711533	Uncultured proteobacterium clone SIMO-2167 16S ribosomal RNA gene, partial sequence	uncultured proteobacterium	Observatory Dean Creek Marsh sampling site
DQ138957	Uncultured bacterium clone JG135 16S ribosomal RNA gene, partial sequence	uncultured bacterium	chemical fertilizer paddy soil
AJ009448	uncultured bacterium SJA-4 16S rRNA gene, clone SJA-4	uncultured bacterium SJA-4	
AB043854	Bacillus sp. N6 gene for 16S rRNA.	Bacillus sp. N6	
AB060974	Rhodococcus opacus gene for 16S rRNA, partial sequence.	Rhodococcus opacus	
AB087523	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence,	uncultured bacterium	activated sludge from lab-scale
AB089951	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	isolated from a filter material of
AB099988	Uncultured bacterium gene for 16S rRNA, partial sequences, clone:	uncultured bacterium	inactive deep-sea hydrothermal vent
AB110635	Sphingomonas sp. MD-1 gene for 16S rRNA, partial sequence.	Sphingomonas sp. MD-1	
AB116121	Bacillus mycoides gene for 16S ribosomal RNA, partial sequence,	Bacillus mycoides	Leaf mold
AB177192	Uncultured bacterium gene for 16S rRNA, clone: ODP1230B3.18.	uncultured bacterium	PCR-derived sequence from methane
AB177205	Uncultured bacterium gene for 16S rRNA, clone: ODP1230B32.09.	uncultured bacterium	PCR-derived sequence from methane
AB177205	Uncultured bacterium gene for 16S rRNA, clone: ODP1230B32.09.	uncultured bacterium	PCR-derived sequence from methane
AB177205	Uncultured bacterium gene for 16S rRNA, clone: ODP1230B32.09.	uncultured bacterium	PCR-derived sequence from methane
AB177205	Uncultured bacterium gene for 16S rRNA, clone: ODP1230B32.09.	uncultured bacterium	PCR-derived sequence from methane
AB177205	Uncultured bacterium gene for 16S rRNA, clone: ODP1230B32.09.	uncultured bacterium	PCR-derived sequence from methane
AB177255	Uncultured bacterium gene for 16S rRNA, clone: ODP1244B5.9.	uncultured bacterium	PCR-derived sequence from methane
AB177319	Uncultured bacterium gene for 16S rRNA, clone: ODP1251B15.18.	uncultured bacterium	PCR-derived sequence from methane
AB177334	Uncultured bacterium gene for 16S rRNA, clone: ODP1251B3.18.	uncultured bacterium	PCR-derived sequence from methane
AB179670	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	obtained from groundwater, 0.2
AB179676	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	obtained from groundwater, 0.2
AB185003	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	activated sludge from intermittent
AB186806	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	uncultured clone from polychlorinated
AB187506	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	uncultured clone from polychlorinated
AB187912	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from compost
AB201587	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence,	uncultured bacterium	obtained from the experimental field at
AB201621	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence,	uncultured bacterium	obtained from the experimental field at
AB234247	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	sediment and soil
AB234247	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	sediment and soil
AB234248	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	sediment and soil
AB234261	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	sediment and soil
AB234266	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	sediment and soil
AB234280	Uncultured bacterium gene for 16S rRNA, partial sequence,	uncultured bacterium	sediment and soil
AB238029	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR product of 16S rRNA gene from
AB240225	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from rhizosphere
AB240264	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from bulk soil of
AB240266	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from bulk soil of
AB240347	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from rhizosphere
AB240347	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from rhizosphere
AB240358	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from rhizosphere
AB240474	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from root-tip (0
AB240491	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from root-tip (0
AB240510	Uncultured bacterium gene for 16S rRNA, partial sequence, clone:	uncultured bacterium	PCR-derived sequence from root-base (80
AB245336	Solirubrobacter sp. Gsoil 921 gene for 16S rRNA, partial sequence.	Solirubrobacter sp. Gsoil 921	soil of the ginseng field
AJ233911	Angiococcus disciformis 16S rRNA gene, strain An d6.	Angiococcus disciformis	
AF013558	Uncultivated soil bacterium clone S105 16S ribosomal RNA gene,	uncultivated soil bacterium clone S105	
AF013558	Uncultivated soil bacterium clone S105 16S ribosomal RNA gene,	uncultivated soil bacterium clone S105	
AF141536	Uncultured firmicute clone CRE-PA64 16S ribosomal RNA gene, partial	uncultured Gram-positive bacterium	
AF280847	Uncultured bacterium mle1-8 16S ribosomal RNA gene, partial	uncultured bacterium mle1-8	
AF292999	Uncultured Green Bay ferromanganous micronodule bacterium MND8 16S	uncultured Green Bay ferromanganous micronodule bacterium MND8	
AF316785	Uncultured Crater Lake bacterium CL500-26 16S ribosomal RNA gene,	uncultured Crater Lake bacterium CL500-26	
AF316785	Uncultured Crater Lake bacterium CL500-26 16S ribosomal RNA gene,	uncultured Crater Lake bacterium CL500-26	
AF317771	Unidentified bacterium wb1_N15 small subunit ribosomal RNA gene,	unidentified bacterium wb1_N15	
AF320959	Uncultured Lake Michigan sediment bacterium LMBA44 16S ribosomal	uncultured Lake Michigan sediment bacterium LMBA44	
AF320959	Uncultured Lake Michigan sediment bacterium LMBA44 16S ribosomal	uncultured Lake Michigan sediment bacterium LMBA44	
AF351238	Uncultured delta proteobacterium clone 8-45 16S ribosomal RNA gene,	uncultured delta proteobacterium	
AF388341	Uncultured bacterium clone Ac16 16S ribosomal RNA gene, partial	uncultured bacterium	
AF388362	Uncultured bacterium clone Ac57 16S ribosomal RNA gene, partial	uncultured bacterium	
AF388362	Uncultured bacterium clone Ac57 16S ribosomal RNA gene, partial	uncultured bacterium	
AF392798	Uncultured bacterium clone CDG5 16S ribosomal RNA gene, partial	uncultured bacterium	
AF407200	Uncultured bacterium clone GIF9 16S ribosomal RNA gene, partial	uncultured bacterium	
AF422593	Uncultured bacterium clone t019 16S ribosomal RNA gene, partial	uncultured bacterium	
AF422607	Uncultured bacterium clone t037 16S ribosomal RNA gene, partial	uncultured bacterium	
AF422607	Uncultured bacterium clone t037 16S ribosomal RNA gene, partial	uncultured bacterium	

Version	DEFINITION	SOURCE	isolation_source
AF423245	Uncultured soil bacterium clone 288-2 16S ribosomal RNA gene,	uncultured soil bacterium	
AF443586	Uncultured bacterium clone C-F-15 16S ribosomal RNA gene, partial	uncultured bacterium	
AF446261	Uncultured firmicute FL08D08 16S ribosomal RNA gene, partial	uncultured firmicute	
AF495444	Uncultured eubacterium clone F13.6 16S ribosomal RNA gene, partial	uncultured bacterium	sludge of TaeJon WWTP
AF523321	Uncultured bacterium clone P4-55 16S ribosomal RNA gene, partial	uncultured bacterium	sediment at an inactive uranium mine
AF523332	Uncultured bacterium clone P4-1 16S ribosomal RNA gene, partial	uncultured bacterium	sediment at an inactive uranium mine
AJ585959	Thermococcales archaeon T30a-17 partial 16S rRNA gene, clone	Thermococcales archaeon T30a-17	enrichment culture from hydrothermal
AJ617866	Uncultured bacterium 16S rRNA gene, clone D14422.	uncultured bacterium	oxic-anoxic interphase of flooded paddy
AJ704365	Uncultured bacterium partial 16S rRNA gene, clone IMB1.	uncultured bacterium	sulfidic surface waters from whitish
AJ745078	Hypoponera opacior microsatellite DNA, locus HoP60.	Hypoponera opacior	
AJ784135	Vibrio sp. K3-01 partial 16S rRNA gene.	Vibrio sp. K3-01	surface-sterilised Amberjack, Seriola
AJ853514	Uncultured bacterium partial 16S rRNA gene, clone GZKB19.	uncultured bacterium	landfill leachate
AJ853514	Uncultured bacterium partial 16S rRNA gene, clone GZKB19.	uncultured bacterium	landfill leachate
AJ853599	Uncultured bacterium partial 16S rRNA gene, clone GZKB106.	uncultured bacterium	landfill leachate
AJ853938	Uncultured bacterium partial 16S rRNA gene, clone A44.	uncultured bacterium	originally, natural surface water
AJ863184	Uncultured bacterium partial 16S rRNA gene, clone 20BSU24.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863185	Uncultured bacterium partial 16S rRNA gene, clone 25BSU46.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863186	Uncultured bacterium partial 16S rRNA gene, clone 20BSU14.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863189	Uncultured bacterium partial 16S rRNA gene, clone 20BSU39.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863206	Uncultured bacterium partial 16S rRNA gene, clone 25BSU8.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863208	Uncultured bacterium partial 16S rRNA gene, clone 25BSU20.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863216	Uncultured bacterium partial 16S rRNA gene, clone 20BSU60.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863232	Uncultured bacterium partial 16S rRNA gene, clone 26BSF26.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863233	Uncultured bacterium partial 16S rRNA gene, clone 26BSF35.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863236	Uncultured bacterium partial 16S rRNA gene, clone 26BSF29.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863236	Uncultured bacterium partial 16S rRNA gene, clone 26BSF29.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ863242	Uncultured bacterium partial 16S rRNA gene, clone 21BSF23.	uncultured bacterium	poplar tree microcosm, bulk soil,
AJ875423	Bacillus sp. YHWG-15 partial 16S rRNA gene, strain YHWG-15.	Bacillus sp. YHWG-15	reed epiphyton
AJ876729	Uncultured bacterium partial 16S rRNA gene, clone R1_16.	uncultured bacterium	river sediment
AJ888558	Uncultured alphaproteobacterium partial 16S rRNA gene, clone TH433.	uncultured alpha proteobacterium	lake sediment
AJ890100	gamma proteobacterium endosymbiont 1 of Inanidrilus leukodermatus	gamma proteobacterium endosymbiont 1 of Ina	marine sediment
AM159243	Uncultured Bacteroidetes bacterium 16S rRNA gene, clone HrhB26.	uncultured Bacteroidetes bacterium	rice rhizosphere
AM159269	Uncultured Acidobacteriaceae bacterium 16S rRNA gene, clone HrhB54.	uncultured Acidobacteriaceae bacterium	rice rhizosphere
AM159379	Uncultured Bacteroidetes bacterium 16S rRNA gene, clone LrhB75.	uncultured Bacteroidetes bacterium	rice rhizosphere
AM159379	Uncultured Bacteroidetes bacterium 16S rRNA gene, clone LrhB75.	uncultured Bacteroidetes bacterium	rice rhizosphere
AM159457	Uncultured bacterium 16S rRNA gene, clone RB90-58.	uncultured bacterium	rice rhizosphere
AM159457	Uncultured bacterium 16S rRNA gene, clone RB90-58.	uncultured bacterium	rice rhizosphere
AM167966	Uncultured candidate division OP11 bacterium partial 16S rRNA gene,	uncultured candidate division OP11 bacterium	mineral spring
AM167972	Uncultured candidate division OP11 bacterium partial 16S rRNA gene,	uncultured candidate division OP11 bacterium	mineral spring
AJ252611	Agricultural soil bacterium clone SC-I-7, 16S rRNA gene (partial).	agricultural soil bacterium SC-I-7	
AJ252644	Agricultural soil bacterium clone SC-I-60, 16S rRNA gene (partial).	agricultural soil bacterium SC-I-60	
AJ252662	Agricultural soil bacterium clone SC-I-86, 16S rRNA gene (partial).	agricultural soil bacterium SC-I-86	
AJ551170	Arthrobacter sp. An32 partial 16S rRNA gene, isolate An32.	Arthrobacter sp. An32	deep sea sediment
AY013611	Uncultured Banisveld landfill bacterium BVA74a 16S ribosomal RNA	uncultured Banisveld landfill bacterium BVA74a	sample taken from beneath the landfill
AY037562	Uncultured soil bacterium clone S012 16S ribosomal RNA gene,	uncultured soil bacterium	
AY037562	Uncultured soil bacterium clone S012 16S ribosomal RNA gene,	uncultured soil bacterium	
AY037562	Uncultured soil bacterium clone S012 16S ribosomal RNA gene,	uncultured soil bacterium	
AY037562	Uncultured soil bacterium clone S012 16S ribosomal RNA gene,	uncultured soil bacterium	
AY043899	Uncultured actinobacterium clone SMS9.30WL 16S ribosomal RNA gene,	uncultured actinobacterium	
AY043899	Uncultured actinobacterium clone SMS9.30WL 16S ribosomal RNA gene,	uncultured actinobacterium	
AY043899	Uncultured actinobacterium clone SMS9.30WL 16S ribosomal RNA gene,	uncultured actinobacterium	
AY043904	Uncultured actinobacterium clone SMS9.6WL 16S ribosomal RNA gene,	uncultured actinobacterium	
AY043947	Uncultured Verrucomicrobia bacterium clone SMS9.68WL 16S ribosomal	uncultured Verrucomicrobia bacterium	
AY081988	Uncultured bacterium clone KRA30-08 16S ribosomal RNA gene, partial	uncultured bacterium	atrazine-catabolizing microbial
AY135927	Uncultured bacterium clone SG2-137 16S ribosomal RNA gene, partial	uncultured bacterium	
AY144276	Uncultured Flavobacterium sp. LTUCFB05114 16S ribosomal RNA gene,	uncultured Flavobacterium sp.	petroleum-contaminated sandy soil
AY150868	Uncultured Rubrobacteridae bacterium clone glen99_21 16S ribosomal	uncultured Rubrobacteridae bacterium	disturbed surface soil
AY150879	Uncultured Rubrobacteridae bacterium clone glen99_17 16S ribosomal	uncultured Rubrobacteridae bacterium	disturbed surface soil
AY150879	Uncultured Rubrobacteridae bacterium clone glen99_17 16S ribosomal	uncultured Rubrobacteridae bacterium	disturbed surface soil
AY150879	Uncultured Rubrobacteridae bacterium clone glen99_17 16S ribosomal	uncultured Rubrobacteridae bacterium	disturbed surface soil
AY154558	Uncultured earthworm intestine bacterium clone lw63 16S ribosomal	uncultured earthworm intestine bacterium	earthworm intestine
AY154623	Uncultured earthworm cast bacterium clone c273 16S ribosomal RNA	uncultured earthworm cast bacterium	earthworm cast
AY162061	Beta proteobacterium PII_GH1.2.B8 small subunit ribosomal RNA gene,	beta proteobacterium PII_GH1.2.B8	soil
AY162061	Beta proteobacterium PII_GH1.2.B8 small subunit ribosomal RNA gene,	beta proteobacterium PII_GH1.2.B8	soil
AY162061	Beta proteobacterium PII_GH1.2.B8 small subunit ribosomal RNA gene,	beta proteobacterium PII_GH1.2.B8	soil
AY177763	Uncultured Gram-positive bacterium isolate 5G01 16S ribosomal RNA	uncultured Gram-positive bacterium	vadose material found four meters below
AY177765	Uncultured low G+C Gram-positive bacterium 16S ribosomal RNA gene,	uncultured low G+C Gram-positive bacterium	vadose material found four meters below
AY186808	Uncultured bacterium clone unel16 16S ribosomal RNA gene, partial	uncultured bacterium	soil
AY186863	Uncultured bacterium clone uvel18 16S ribosomal RNA gene, partial	uncultured bacterium	soil

Version	DEFINITION	SOURCE	isolation_source
AY214805	Uncultured alpha proteobacterium clone BB-2-G9R 16S ribosomal RNA	uncultured alpha proteobacterium	soil
AY221057	Uncultured bacterium clone CCM22a 16S ribosomal RNA gene, partial	uncultured bacterium	sediments collected at Charon's Cascade
AY221065	Uncultured bacterium clone CCM5b 16S ribosomal RNA gene, partial	uncultured bacterium	sediments collected at Charon's Cascade
AY221611	Uncultured bacterium clone HS9-50 16S ribosomal RNA gene, partial	uncultured bacterium	metal and hydrocarbon contaminated
AY221613	Uncultured soil bacterium clone HS9-66 16S ribosomal RNA gene,	uncultured soil bacterium	metal and hydrocarbon contaminated
AY221615	Uncultured soil bacterium clone HS9-75 16S ribosomal RNA gene,	uncultured soil bacterium	metal and hydrocarbon contaminated
AY221615	Uncultured soil bacterium clone HS9-75 16S ribosomal RNA gene,	uncultured soil bacterium	metal and hydrocarbon contaminated
AY221615	Uncultured soil bacterium clone HS9-75 16S ribosomal RNA gene,	uncultured soil bacterium	metal and hydrocarbon contaminated
AY221615	Uncultured soil bacterium clone HS9-75 16S ribosomal RNA gene,	uncultured soil bacterium	metal and hydrocarbon contaminated
AY235435	Uncultured soil bacterium clone z12 16S ribosomal RNA gene, partial	uncultured soil bacterium	400 mg/kg zinc-contaminated
AY283125	Uncultured low G+C Gram-positive bacterium clone NS-56 16S	uncultured low G+C Gram-positive bacterium	marine sediment
AY307861	Uncultured actinobacterium clone BF-F08 16S ribosomal RNA gene,	uncultured actinobacterium	estuarine sediment
AY326608	Uncultured soil bacterium clone 597-1 small subunit ribosomal RNA	uncultured soil bacterium	soil
AY354168	Uncultured bacterium clone pIR3BB12 16S ribosomal RNA gene, partial	uncultured bacterium	Rainbow hydrothermal vent sediments
AY354188	Uncultured bacterium clone pIR3BG12 16S ribosomal RNA gene, partial	uncultured bacterium	Rainbow hydrothermal vent sediments
AY360666	Uncultured Acidobacteria bacterium clone M10Ba79 small subunit	uncultured Acidobacteria bacterium	oxic rice field soil
AY360666	Uncultured Acidobacteria bacterium clone M10Ba79 small subunit	uncultured Acidobacteria bacterium	oxic rice field soil
AY360666	Uncultured Acidobacteria bacterium clone M10Ba79 small subunit	uncultured Acidobacteria bacterium	oxic rice field soil
AY387299	Uncultured actinobacterium clone 7-248 16S ribosomal RNA gene,	uncultured actinobacterium	Lope tropical rainforest soils
AY395377	Uncultured Rubrobacteridae bacterium clone EB1058 16S ribosomal RNA	uncultured Rubrobacteridae bacterium	pasture soil
AY395438	Uncultured Rubrobacteridae bacterium clone EB1119 16S ribosomal RNA	uncultured Rubrobacteridae bacterium	pasture soil
AY425772	Uncultured bacterium clone 1790-4 16S ribosomal RNA gene, partial	uncultured bacterium	volcanic deposit from 1790
AY456903	Uncultured bacterium clone DBW1-51 16S ribosomal RNA gene, partial	uncultured bacterium	Dover Air Force Base below water table
AY464463	Pseudomonas sp. ACP14 small subunit ribosomal RNA gene, partial	Pseudomonas sp. ACP14	
AY464548	Thermomonosporaceae bacterium CNR431 small subunit ribosomal RNA	Thermomonosporaceae bacterium CNR431	marine sediment
AY464548	Thermomonosporaceae bacterium CNR431 small subunit ribosomal RNA	Thermomonosporaceae bacterium CNR431	marine sediment
AY493917	Uncultured soil bacterium clone 439 small subunit ribosomal RNA	uncultured soil bacterium	soil
AY493917	Uncultured soil bacterium clone 439 small subunit ribosomal RNA	uncultured soil bacterium	soil
AY493936	Uncultured soil bacterium clone 1508 small subunit ribosomal RNA	uncultured soil bacterium	soil
AY494658	Uncultured Conexibacter sp. clone ACTINO10 16S ribosomal RNA gene,	uncultured Conexibacter sp.	Salmo salar gill
AY508257	Uncultured bacterium clone HC21_10 16S ribosomal RNA gene, complete	uncultured bacterium	microbial mat
AY515486	Uncultured Bacteroidetes bacterium clone GWS-Kdna25 16S ribosomal	uncultured Bacteroidetes bacterium	bulk water of the German Wadden Sea,
AY542229	Uncultured Chloroflexi bacterium clone GoM IDB-24 16S ribosomal RNA	uncultured Chloroflexi bacterium	Gulf of Mexico seafloor sediments
AY546507	Uncultured bacterium clone 5H_28 16S ribosomal RNA gene, partial	uncultured bacterium	soda lake sediment
AY546507	Uncultured bacterium clone 5H_28 16S ribosomal RNA gene, partial	uncultured bacterium	soda lake sediment
AY546507	Uncultured bacterium clone 5H_28 16S ribosomal RNA gene, partial	uncultured bacterium	soda lake sediment
AY555810	Uncultured bacterium clone PK350 16S ribosomal RNA gene, partial	uncultured bacterium	Bor Khlueng hot spring
AY568514	Burkholderia sp. SE-10 16S ribosomal RNA gene, partial sequence.	Burkholderia sp. SE-10	
AY568514	Burkholderia sp. SE-10 16S ribosomal RNA gene, partial sequence.	Burkholderia sp. SE-10	
AY568514	Burkholderia sp. SE-10 16S ribosomal RNA gene, partial sequence.	Burkholderia sp. SE-10	
AY568514	Burkholderia sp. SE-10 16S ribosomal RNA gene, partial sequence.	Burkholderia sp. SE-10	
AY568514	Burkholderia sp. SE-10 16S ribosomal RNA gene, partial sequence.	Burkholderia sp. SE-10	
AY568768	Uncultured bacterium isolate JH10_C09 16S ribosomal RNA gene,	uncultured bacterium	
AY568857	Uncultured bacterium isolate JH12_C15 16S ribosomal RNA gene,	uncultured bacterium	
AY568907	Uncultured bacterium isolate JH12_C69 16S ribosomal RNA gene,	uncultured bacterium	
AY568908	Uncultured bacterium isolate JH12_C70 16S ribosomal RNA gene,	uncultured bacterium	
AY569777	Cloning vector pZero++ Kan, complete sequence.	Cloning vector pZero++ Kan	
AY570583	Uncultured bacterium clone PL-14B8 16S ribosomal RNA gene, partial	uncultured bacterium	low-temperature biodegraded Canadian
AY584744	Uncultured bacterium clone JWBH-3 16S ribosomal RNA gene, partial	uncultured bacterium	Chesapeake Bay Watershed
AY592366	Uncultured bacterium clone Amsterdam-2B-06 16S ribosomal RNA gene,	uncultured bacterium	Amsterdam mud volcano, Eastern
AY592619	Uncultured bacterium clone Napoli-1B-64 16S ribosomal RNA gene,	uncultured bacterium	Napoli mud volcano, Eastern
AY607163	Uncultured bacterium clone X9Ba17 small subunit ribosomal RNA gene,	uncultured bacterium	anoxic rice field soil
AY607176	Uncultured Clostridia bacterium clone X9Ba34 small subunit	uncultured Clostridia bacterium	anoxic rice field soil
AY632433	Uncultured alpha proteobacterium clone E1-6 16S ribosomal RNA gene,	uncultured alpha proteobacterium	Biosphere 2 soil
AY694491	Uncultured Sphingobacteriales bacterium clone JAB SHC 110 16S	uncultured Sphingobacteriales bacterium	soil
AY694600	Uncultured bacterium clone JAB SMS 55 16S ribosomal RNA gene,	uncultured bacterium	soil
AY694604	Uncultured Verrucomicrobia bacterium clone JAB SMS 61 16S ribosomal	uncultured Verrucomicrobia bacterium	soil
AY694610	Uncultured Verrucomicrobia bacterium clone JAB SMS 86 16S ribosomal	uncultured Verrucomicrobia bacterium	soil
AY710627	Uncultured bacterium clone SIMO-1187 16S ribosomal RNA gene,	uncultured bacterium	lon=81.2797W, lat=31.3884N; sediment
AY725249	Uncultured bacterium clone S1-1-CL12 16S ribosomal RNA gene,	uncultured bacterium	decayed velvetleaf seed
AY754024	Phagemid vector pMID21, complete sequence.	Phagemid vector pMID21	
AY834304	Uncultured bacterium clone cloRDC-25 16S ribosomal RNA gene,	uncultured bacterium	potato rhizosphere
AY834311	Uncultured bacterium clone cloRDC-42 16S ribosomal RNA gene,	uncultured bacterium	potato rhizosphere
AY834348	Uncultured bacterium clone cloRDL-22 16S ribosomal RNA gene,	uncultured bacterium	potato rhizosphere
AY869683	Uncultured bacterium clone FS117-41B-02 16S ribosomal RNA gene,	uncultured bacterium	ridge flank crustal fluids
AY869683	Uncultured bacterium clone FS117-41B-02 16S ribosomal RNA gene,	uncultured bacterium	ridge flank crustal fluids
AY869683	Uncultured bacterium clone FS117-41B-02 16S ribosomal RNA gene,	uncultured bacterium	ridge flank crustal fluids
AY869683	Uncultured bacterium clone FS117-41B-02 16S ribosomal RNA gene,	uncultured bacterium	ridge flank crustal fluids
AY917287	Uncultured bacterium clone 1700a2-04 16S ribosomal RNA gene,	uncultured bacterium	volcanic deposit from 1700

Version	DEFINITION	SOURCE	isolation_source
AY917420	Uncultured bacterium clone 1700a-24 16S ribosomal RNA gene, partial	uncultured bacterium	volcanic deposit from 1700
AY917425	Uncultured bacterium clone 1700a-31 16S ribosomal RNA gene, partial	uncultured bacterium	volcanic deposit from 1700
AY917847	Uncultured bacterium clone 1974a-10 16S ribosomal RNA gene, partial	uncultured bacterium	volcanic deposit from 1974
AY921485	Uncultured eubacterium clone AP10 16S ribosomal RNA gene, partial	uncultured bacterium	rice field soil
AY921569	Uncultured eubacterium clone A30 16S ribosomal RNA gene, partial	uncultured bacterium	rice field soil
AY921654	Uncultured alpha proteobacterium clone AKYG1791 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY921703	Uncultured beta proteobacterium clone AKYG1828 16S ribosomal RNA	uncultured beta proteobacterium	farm soil adjacent to a silage storage
AY921704	Uncultured Gemmatimonadetes bacterium clone AKYH530 16S ribosomal	uncultured Gemmatimonadetes bacterium	farm soil adjacent to a silage storage
AY921769	Uncultured beta proteobacterium clone AKYG1037 16S ribosomal RNA	uncultured beta proteobacterium	farm soil adjacent to a silage storage
AY921821	Uncultured beta proteobacterium clone AKYH490 16S ribosomal RNA	uncultured beta proteobacterium	farm soil adjacent to a silage storage
AY921830	Uncultured delta proteobacterium clone AKYH1112 16S ribosomal RNA	uncultured delta proteobacterium	farm soil adjacent to a silage storage
AY921838	Uncultured Acidobacteria bacterium clone AKYH1421 16S ribosomal RNA	uncultured Acidobacteria bacterium	farm soil adjacent to a silage storage
AY921838	Uncultured Acidobacteria bacterium clone AKYH1421 16S ribosomal RNA	uncultured Acidobacteria bacterium	farm soil adjacent to a silage storage
AY921859	Uncultured Gemmatimonadetes bacterium clone AKYH1258 16S ribosomal	uncultured Gemmatimonadetes bacterium	farm soil adjacent to a silage storage
AY921867	Uncultured Chloroflexi bacterium clone AKYG475 16S ribosomal RNA	uncultured Chloroflexi bacterium	farm soil adjacent to a silage storage
AY921881	Uncultured Acidobacteria bacterium clone AKYH1176 16S ribosomal RNA	uncultured Acidobacteria bacterium	farm soil adjacent to a silage storage
AY921904	Uncultured Chloroflexi bacterium clone AKYG999 16S ribosomal RNA	uncultured Chloroflexi bacterium	farm soil adjacent to a silage storage
AY921908	Uncultured Chloroflexi bacterium clone AKYG799 16S ribosomal RNA	uncultured Chloroflexi bacterium	farm soil adjacent to a silage storage
AY921913	Uncultured Chloroflexi bacterium clone AKYH513 16S ribosomal RNA	uncultured Chloroflexi bacterium	farm soil adjacent to a silage storage
AY921916	Uncultured alpha proteobacterium clone AKYG1580 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY921916	Uncultured alpha proteobacterium clone AKYG1580 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY921916	Uncultured alpha proteobacterium clone AKYG1580 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY921916	Uncultured alpha proteobacterium clone AKYG1580 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY921916	Uncultured alpha proteobacterium clone AKYG1580 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY921916	Uncultured alpha proteobacterium clone AKYG1580 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY921916	Uncultured alpha proteobacterium clone AKYG1580 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY921940	Uncultured Actinobacteria bacterium clone AKYG950 16S ribosomal RNA	uncultured actinobacterium	farm soil adjacent to a silage storage
AY921949	Uncultured candidate division SPAM bacterium clone AKYG1047 16S	uncultured candidate division SPAM bacterium	farm soil adjacent to a silage storage
AY922024	Uncultured Actinobacteria bacterium clone AKYG476 16S ribosomal RNA	uncultured actinobacterium	farm soil adjacent to a silage storage
AY922084	Uncultured delta proteobacterium clone AKYG984 16S ribosomal RNA	uncultured delta proteobacterium	farm soil adjacent to a silage storage
AY922126	Uncultured alpha proteobacterium clone AKYH1530 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY922150	Uncultured Acidobacteria bacterium clone AKYH707 16S ribosomal RNA	uncultured Acidobacteria bacterium	farm soil adjacent to a silage storage
AY922159	Uncultured alpha proteobacterium clone AKYH831 16S ribosomal RNA	uncultured alpha proteobacterium	farm soil adjacent to a silage storage
AY947554	Sphingomonas sp. DB-1 16S ribosomal RNA gene, partial sequence.	Sphingomonas sp. DB-1	
AY953235	Uncultured anaerobic bacterium clone A-3E 16S ribosomal RNA gene,	uncultured anaerobic bacterium	anaerobic swine lagoon
AY955095	Uncultured bacterium clone 69-7G 16S ribosomal RNA gene, partial	uncultured bacterium	sediment
AY955095	Uncultured bacterium clone 69-7G 16S ribosomal RNA gene, partial	uncultured bacterium	sediment
AY957930	Uncultured bacterium clone B3NR69D15 16S ribosomal RNA gene,	uncultured bacterium	drinking water biofilm
AY959162	Uncultured bacterium clone rRNA389 16S ribosomal RNA gene, partial	uncultured bacterium	human vaginal epithelium
AY988887	Uncultured soil bacterium clone L1A.5A08 16S ribosomal RNA gene,	uncultured soil bacterium	soil
AY989063	Uncultured soil bacterium clone L1A.7F03 16S ribosomal RNA gene,	uncultured soil bacterium	soil
AY989545	Uncultured soil bacterium clone L1A.14D08 16S ribosomal RNA gene,	uncultured soil bacterium	soil
Z95708	Bacterial species 16S rRNA gene (clone 11-24).	Bacteria (eubacteria)	
Y07580	Uncultured bacterium DA011 partial 16S rRNA gene.	uncultured bacterium DA011	isolated from Drentse A grassland soil
AJ506120	Clostridium bowmanii 16S rRNA gene, type strain DSM 14206, clone	Clostridium bowmanii	
AJ544074	Crassostrea gigas tbetaRI gene for TGF-beta Type I receptor, exons	Crassostrea gigas (Pacific oyster)	
AJ544074	Crassostrea gigas tbetaRI gene for TGF-beta Type I receptor, exons	Crassostrea gigas (Pacific oyster)	
AJ544074	Crassostrea gigas tbetaRI gene for TGF-beta Type I receptor, exons	Crassostrea gigas (Pacific oyster)	
AJ544074	Crassostrea gigas tbetaRI gene for TGF-beta Type I receptor, exons	Crassostrea gigas (Pacific oyster)	
CR933146	Uncultured bacterium partial 16S rRNA gene from clone	uncultured bacterium	Evry municipal wastewater treatment
DQ018805	Uncultured anaerobic bacterium clone C-125 16S ribosomal RNA gene,	uncultured anaerobic bacterium	anaerobic swine lagoon
DQ058675	Uncultured bacterium clone Biofilm_256d_c12 16S ribosomal RNA gene,	uncultured bacterium	drinking water distribution system
DQ065070	Uncultured freshwater bacterium clone 965019H11.x1 16S ribosomal	uncultured freshwater bacterium	freshwater
DQ066981	Uncultured bacterium clone pLW-101 16S ribosomal RNA gene, partial	uncultured bacterium	sediment of Lake Washington
DQ066989	Uncultured bacterium clone pLW-53 16S ribosomal RNA gene, partial	uncultured bacterium	sediment of Lake Washington
DQ067007	Uncultured bacterium clone pLW-88 16S ribosomal RNA gene, partial	uncultured bacterium	sediment of Lake Washington
DQ067029	Uncultured bacterium clone pLW-45 16S ribosomal RNA gene, partial	uncultured bacterium	sediment of Lake Washington
DQ076455	Uncultured Rhodoferax sp. clone TD-46 16S ribosomal RNA gene,	uncultured Rhodoferax sp.	glacier ice
DQ083105	Uncultured bacterium clone X20 16S ribosomal RNA gene, partial	uncultured bacterium	soil
DQ088792	Uncultured bacterium clone MP104-SW-b11 16S ribosomal RNA gene,	uncultured bacterium	crustal biotome
DQ093903	Uncultured bacterium clone ga34 16S ribosomal RNA gene, partial	uncultured bacterium	rhizosphere
DQ093903	Uncultured bacterium clone ga34 16S ribosomal RNA gene, partial	uncultured bacterium	rhizosphere
DQ093903	Uncultured bacterium clone ga34 16S ribosomal RNA gene, partial	uncultured bacterium	rhizosphere
DQ093926	Uncultured bacterium clone ga61 16S ribosomal RNA gene, partial	uncultured bacterium	rhizosphere
DQ093934	Uncultured bacterium clone ga71 16S ribosomal RNA gene, partial	uncultured bacterium	rhizosphere
DQ093937	Uncultured bacterium clone ga74 16S ribosomal RNA gene, partial	uncultured bacterium	rhizosphere
DQ093950	Uncultured bacterium clone ga88 16S ribosomal RNA gene, partial	uncultured bacterium	rhizosphere
DQ108394	Brevundimonas sp. Tibet-IBa1 16S ribosomal RNA gene, partial	Brevundimonas sp. Tibet-IBa1	Qinghai-Tibet Plateau permafrost
DQ110117	Uncultured alpha proteobacterium clone 406T3 16S ribosomal RNA	uncultured alpha proteobacterium	

Version	DEFINITION	SOURCE	isolation_source
DQ110128	Uncultured bacterium clone 451T3 16S ribosomal RNA gene, partial	uncultured bacterium	
DQ123789	Uncultured soil bacterium clone PAH-Feed-65 16S ribosomal RNA gene,	uncultured soil bacterium	PAH-contaminated soil
DQ125648	Uncultured bacterium clone AKAU3697 16S ribosomal RNA gene, partial	uncultured bacterium	uranium contaminated soil
DQ125650	Uncultured bacterium clone AKAU3700 16S ribosomal RNA gene, partial	uncultured bacterium	uranium contaminated soil
DQ125669	Uncultured bacterium clone AKAU3738 16S ribosomal RNA gene, partial	uncultured bacterium	uranium contaminated soil
DQ125814	Uncultured bacterium clone AKAU3960 16S ribosomal RNA gene, partial	uncultured bacterium	uranium contaminated soil
DQ128365	Uncultured soil bacterium clone HSB CT53_B11 16S ribosomal RNA	uncultured soil bacterium	HSB conventional tillage soil
DQ128372	Uncultured soil bacterium clone HSB CT53_E08 16S ribosomal RNA	uncultured soil bacterium	HSB conventional tillage soil
DQ128736	Uncultured soil bacterium clone HSB NT22_H12 16S ribosomal RNA	uncultured soil bacterium	HSB no tillage soil
DQ128781	Uncultured soil bacterium clone HSB NT53_A10 16S ribosomal RNA	uncultured soil bacterium	HSB no tillage soil
DQ128791	Uncultured soil bacterium clone HSB NT53_B11 16S ribosomal RNA	uncultured soil bacterium	HSB no tillage soil
DQ128791	Uncultured soil bacterium clone HSB NT53_B11 16S ribosomal RNA	uncultured soil bacterium	HSB no tillage soil
DQ128951	Uncultured soil bacterium clone HSB OF51_B04 16S ribosomal RNA	uncultured soil bacterium	HSB old forest soil
DQ129016	Uncultured soil bacterium clone HSB OF53_D09RU 16S ribosomal RNA	uncultured soil bacterium	HSB old forest soil
DQ129053	Uncultured soil bacterium clone CWT SM01_E06 16S ribosomal RNA	uncultured soil bacterium	Coweeta forest soil
DQ129485	Uncultured bacterium clone AKIW778 16S ribosomal RNA gene, partial	uncultured bacterium	urban aerosol
DQ129631	Uncultured bacterium clone AKIW856 16S ribosomal RNA gene, partial	uncultured bacterium	urban aerosol
DQ138955	Uncultured bacterium clone JG89 16S ribosomal RNA gene, partial	uncultured bacterium	chemical fertilizer paddy soil
DQ145139	Uncultured bacterium clone A41 16S ribosomal RNA gene, partial	uncultured bacterium	wetland
DQ154301	Soil bacterium RFS-I2 16S ribosomal RNA gene, partial sequence.	soil bacterium RFS-I2	Ross Forest soil
DQ154336	Uncultured soil bacterium clone RFS-C5 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154336	Uncultured soil bacterium clone RFS-C5 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154361	Uncultured soil bacterium clone RFS-C33 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154377	Uncultured soil bacterium clone RFS-C50 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154377	Uncultured soil bacterium clone RFS-C50 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154377	Uncultured soil bacterium clone RFS-C50 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154377	Uncultured soil bacterium clone RFS-C50 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154377	Uncultured soil bacterium clone RFS-C50 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154420	Uncultured soil bacterium clone RFS-C94 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154422	Uncultured soil bacterium clone RFS-C96 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154442	Uncultured soil bacterium clone RFS-C117 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154451	Uncultured soil bacterium clone RFS-C128 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154515	Uncultured soil bacterium clone RFS-C198 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154525	Uncultured soil bacterium clone RFS-C208 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154525	Uncultured soil bacterium clone RFS-C208 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154525	Uncultured soil bacterium clone RFS-C208 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154525	Uncultured soil bacterium clone RFS-C208 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154527	Uncultured soil bacterium clone RFS-C210 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154551	Uncultured soil bacterium clone RFS-C237 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154562	Uncultured soil bacterium clone RFS-C248 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154562	Uncultured soil bacterium clone RFS-C248 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154581	Uncultured soil bacterium clone RFS-C267 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154621	Uncultured soil bacterium clone RFS-C308 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154627	Uncultured soil bacterium clone RFS-C314 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154633	Uncultured soil bacterium clone RFS-C321 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154634	Uncultured soil bacterium clone RFS-C322 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154634	Uncultured soil bacterium clone RFS-C322 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ154651	Uncultured soil bacterium clone RFS-C341 16S ribosomal RNA gene,	uncultured soil bacterium	Ross Forest soil
DQ158100	Uncultured bacterium clone 248 16S ribosomal RNA gene, partial	uncultured bacterium	soil
DQ165091	Uncultured bacterium clone 118 16S ribosomal RNA gene, partial	uncultured bacterium	Upper Mystic Lake sediment
DQ165096	Uncultured bacterium clone 126 16S ribosomal RNA gene, partial	uncultured bacterium	Upper Mystic Lake sediment
DQ165096	Uncultured bacterium clone 126 16S ribosomal RNA gene, partial	uncultured bacterium	Upper Mystic Lake sediment
DQ165096	Uncultured bacterium clone 126 16S ribosomal RNA gene, partial	uncultured bacterium	Upper Mystic Lake sediment
DQ165096	Uncultured bacterium clone 126 16S ribosomal RNA gene, partial	uncultured bacterium	Upper Mystic Lake sediment
DQ165096	Uncultured bacterium clone 126 16S ribosomal RNA gene, partial	uncultured bacterium	Upper Mystic Lake sediment
DQ191697	Uncultured bacterium clone AME E27 16S ribosomal RNA gene, partial	uncultured bacterium	anaerobic bioreactor treating
DQ191735	Uncultured bacterium clone AME E18 16S ribosomal RNA gene, partial	uncultured bacterium	anaerobic bioreactor treating
DQ195646	Uncultured bacterium isolate High.2.29.F9.HB28 16S ribosomal RNA	uncultured bacterium	soil
DQ201592	Uncultured Geobacter sp. clone U3A58 16S ribosomal RNA gene,	uncultured Geobacter sp.	
DQ201599	Uncultured Clostridium sp. clone U3A8 16S ribosomal RNA gene,	uncultured Clostridium sp.	
DQ202161	Uncultured bacterium clone CJRC08 16S ribosomal RNA gene, partial	uncultured bacterium	fluidized bed reactor
DQ211504	Uncultured Fusobacteria bacterium clone nsc154 16S ribosomal RNA	uncultured Fusobacteria bacterium	Shimanto River system
DQ223088	Uncultured bacterium clone S4 16S ribosomal RNA gene, partial	uncultured bacterium	chlorinated ethene-contaminated
DQ228372	Uncultured bacterium clone BG.d11 16S ribosomal RNA gene, partial	uncultured bacterium	Bench Glacier
DQ248291	Uncultured soil bacterium clone TD4 16S ribosomal RNA gene, partial	uncultured soil bacterium	carbon tetrachloride contaminated soil
DQ297986	Uncultured soil bacterium clone UC8 16S ribosomal RNA gene, partial	uncultured soil bacterium	hydrocarbon contaminated soil
DQ329344	Bacterium C26 16S ribosomal RNA gene, partial sequence.	bacterium C26	aerotank
DQ335011	Uncultured planctomycete clone DHBANG110 16S ribosomal RNA gene,	uncultured planctomycete	apple orchard soil

Version	DEFINITION	SOURCE	isolation_source
AJ006090	Unidentified eubacterium 16S rRNA gene (clone TBS21).	uncultured bacterium	
PPL252717	Potato plant root bacterium clone RC-III-33, 16S rRNA gene	potato plant root bacterium RC-III-33	
AJ431217	Proteobacterium BHI60-9 16S rRNA gene, strain BHI60-9.	proteobacterium BHI60-9	
ST416168	Uncultured bacterium partial 16S rRNA gene, clone Sta0-45.	uncultured bacterium	
AJ318159	Uncultured Actinobacterium 16S rRNA gene, clone BIIii24b.	uncultured actinobacterium	
AJ306790	Uncultured bacterium partial 16S rRNA gene, clone SHA-59.	uncultured bacterium	
AJ518553	Unidentified bacterium partial 16S rRNA gene, clone Qui4P1-81.	unidentified bacterium	sediment
AJ519644	Uncultured Chlorobi bacterium partial 16S rRNA gene, clone	uncultured Chlorobi bacterium	uranium mill tailings, soil sample
AJ582053	Uncultured bacterium partial 16S rRNA gene, clone KCM-C-23.	uncultured bacterium	Soil sample collected near the
AJ567598	Uncultured delta proteobacterium partial 16S rRNA gene, clone	uncultured delta proteobacterium	deep-sea sediment
AJ005994	Unidentified eubacterium 16S rRNA gene (clone TBS1).	uncultured bacterium	
AJ232848	Unidentified eubacterium 16S rRNA gene (clone LRS12).	uncultured bacterium	
AJ292615	uncultured eubacterium WD282 partial 16S rRNA gene, clone WD282.	uncultured eubacterium WD282	
AJ390466	Uncultured soil bacterium PBS-40 partial 16S rRNA gene.	uncultured soil bacterium PBS-40	

Appendix Y: Sigma DNA Ladder-Directload™ 1KB

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SIGMA®

ProductInformation

DNA LADDER, DIRECTLOAD™, 1 KB
(0.50-10 kb)

Product No. **D 3937**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Suitable for use as an electrophoresis marker for DNA. Supplied as a 250 µg vial in 1x loading buffer containing marker dyes bromophenol blue and xylene cyanol FF. Ready for use in agarose gels.

FRAGMENT SIZES: base pairs (bp)

10,000
8,000
6,000
5,000
4,000
3,000
2,500
2,000
1,500
1,000
500

Reagents
Storage Buffer: Solution in 2.5% Ficoll (Type 400), 0.0125% bromophenol blue, and 0.00625% xylene cyanol FF.

Storage/Stability
This product is shipped at ambient temperature. Marker is stable for 6 months when stored at –20 °C.

Product Profile
Recommended load volume: 5 µl

Number of loads per vial: 100

Adjustments may be made for different well sizes and individual preferences.

Suitability Assay
5 - 10 µl of DNA Ladder DirectLoad™ and 100 - 200 ng of appropriate DNA standards were loaded on a 0.75% (w/v) agarose gel prepared with 1x TBE (Product No. T 9525) running buffer. The gel was run for 2 hours at 70 volts. After ethidium bromide staining, 11 bands (500-10,000 bp) were clearly resolved and the pattern was consistent with the expected fragment sizes.

JLL/JWM 10/03

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14. ABSTRACT

Chlorinated hydrocarbons and their degradation products are among of the most common organic groundwater contaminates in the United States. These compounds attack the central nervous system in animals and can affect the photosynthesis of plants. These compounds are also resistant to degradation in the environment and, because of this, pose a risk to any ecosystem in which they are present. This study identified the dominant microbial species in a constructed treatment wetland at Wright-Patterson AFB, Dayton, Ohio using 16S rRNA gene sequence analysis. Samples were taken from three different depths and during each of the four seasons. These samples were compared with similar samples taken from an uncontaminated, control site located at Valle Greene wetland in Beavercreek, Ohio. The intent of the study was to measure differences between the microbial community of the treatment wetland and the control wetland. It was hypothesized that the bacteria found to degrade the materials in the lab would be present in the treatment wetland and has a higher population than a wetland free of contaminants. This hypothesis would help support the idea that the natural attenuation of chlorinated hydrocarbons is due primarily to biological factors. The study found that the diversity of microbial communities in both the treatment wetlands and control were so great that additional sampling and

15. SUBJECT TERMS

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